PAPER ELECTROPHORESIS

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120 Alexander Street, Princeton, N.J.

257 Fourth Avenue, New York 10, N.Y.

25 Hollinger Road, Toronto 16, Canada

FOR THE BRITISH COMMONWEALTH EXCEPT CANADA:

CLEAVER-HUME PRESS, LTD.

31, Wright's Lane, Kensington, London, W.8.

AN INTRODUCTION TO PAPER ELECTROPHORESIS AND RELATED METHODS

by

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SECOND IMPRESSION
WITH MINOR ADDITIONS

ELSEVIER PUBLISHING COMPANY

AMSTERDAM LONDON NEW YORK PRINCETON

1957

Library of Congress Catalog Card Number: *57—11352*

First published, 1955

Second impression (with minor additions), 1957

PRINTED IN THE NETHERLANDS BY
N.V. DRUKKERIJ KOCH & KNUTTEL, GOUDA

Preface

In the last few years several new separation techniques have been introduced in inorganic and organic chemistry. While there are already a number of good accounts of paper chromatography and ion exchange methods, paper electrophoresis has so far been only mentioned as a chapter in books on chromatography or in rather specialised treatises dealing with protein electrophoresis and its clinical applications.

It was thought that a short book discussing the many techniques of paper electrophoresis employed up to date, and designed for the chemist who requires an introduction to the methods and applications of paper electrophoresis, would be of more use at the moment than a comprehensive review of the whole literature. This book was thus written to be of assistance to workers in the fields of analytical, organic, and inorganic chemistry, radiochemistry, and biochemistry and to show where electrophoresis on paper or columns can be applied to improve or speed up existing separations, especially where no other separation method has been successful, or where only minute quantities are available for study and no extensive analytical equipment is on hand.

As will be seen from the contents, many fields are so far unexplored and hence the principles underlying the separations are explained as fully as possible to aid the reader in working out other separations or in applying existing ones to his special requirements.

The extensive work done on serum proteins and on clinical applications are referred to but not discussed in detail since some monographs dealing more specifically with clinical chemistry are already in existence:

Microélectrophorèse sur papier, by Macheboeuf, Rebeyrotte, Dubert and Brunerie (Paris, 1954).

Die Papierelektrophorese, by Ch. Wunderly (Sauerländer & Co., Aarau, 1954).

However, for general use, references to the work done in clinical chemistry are provided so that the original papers may be consulted.

MICHAEL LEDERER

Paris, March 1955

Preface to second impression

The second impression of this book remains essentially an introduction and is not meant to be a comprehensive treatise of paper electrophoresis. The alterations made are thus mainly descriptions of new techniques that appear to be of general interest. New references were also added to many of the chapters but only work that was of special interest to the author has been inserted in the text.

Since the publication of the first impression in 1955 several new books, colloquia and symposia on paper electrophoresis have been published. Of these we should like to mention:

Ionography, by H. J. McDonald (The Year Book Publishers, Chicago, 1955).

Manuel of Paper Chromatography and Paper Electrophoresis, by R. J. Block, E. L. Durrum and G. Zweig (Academic Press Inc., New York, 1955).

Paper Electrophoresis, A Ciba Foundation Symposium (Churchill, London, 1956).

MICHAEL LEDERER

Paris, October 1956

Acknowledgments

The author would like to express his grateful acknowledgments to the following: to Dr. E. Lederer for his reading of the manuscript and his many helpful suggestions; to the firms Bender and Hobein, Jouan, Shandon, Dr Virus KG and Strübin for kindly providing illustrations and blocks; and to the following authors and journals for their kind permission to reproduce illustrations: J. R. Cannon, R. Consden, E. L. Durrum, A. H. Gordon, F. C. Larson, E. Lederer, G. B. Marini-Bettolo, A. J. P. Martin, H. Michl, Q. P. Peniston, K. G. Rienits, V. Schwarz, N. Siliprandi, J. D. Smith, H. H. Strain, H. Svensson, A. Tiselius, O. Westphal, Th. Wieland, W. C. Wolfson and Ch. Wunderly, and the editors of *Analytica Chimica Acta*, *Acta Chemica Scandinavica*, *Analytical Chemistry*, *Biochemical Journal*, *Chimia*, *Faraday Society Symposia*, *Journal of the American Chemical Society*, *Journal of Clinical Investigation*, *Monatshefte für Chemie*, *Nature*, and *Science*.

M. L.

Contents

1. Introduction

HISTORICAL

The various recent articles on the history of chromatography [1] have only helped to illustrate the impossibility of crediting one worker with the application or discovery of a general phenomenon, such as adsorption analysis or movement of particles or ions in an electric field, and its analytical application. We shall briefly outline the early work here, without attempting a complete survey, and shall state the main systems in which electrophoretic measurements and analyses have been carried out.

Lodge [2] is credited with the first attempt (in 1886) to measure ionic velocities in a tube filled with jelly containing an indicator (e.g. phenolphthalein), as shown in Fig. 1. An acid and an alkali or Ba^{++} and SO_4^{--} ions are

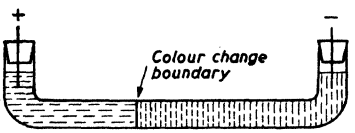

Fig. 1. Apparatus for measurement of ionic velocities after Lodge.

added to the two electrodes, electrolysis carried out and the rate of indicator change or of precipitation measured.

Whetham measured the movement of ions in the free

solution (without stabilising jelly) by the rate at which a coloured ion travels into a colourless solution in an apparatus given in Fig. 2.

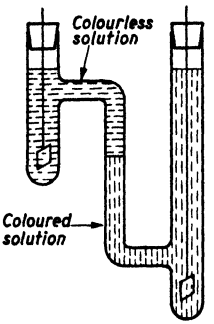

Fig. 2. Apparatus for measurement of ionic velocities in solution after Whetham.

It will be evident, that neither method is capable of precise results, and that no device for an actual separation of substances of different velocities was attempted.

An analytical method based on electrophoresis inside agar jelly was evolved in 1923 by Kendall [3], who successfully separated pairs of rare earths and other metals, and it is surprising that this work was completely forgotten and not taken up by others until very recently.

TISELIUS METHOD

The observation of moving boundaries was converted into an analytical method in 1925 by Tiselius, continuing the work of Svedberg, Jette and Scott.

Tiselius has summarised the evolution of his method in his Nobel prize lecture [4]. The first important step was the development of optical methods, capable of recording quantitatively the change of refractive index of a colourless moving boundary. Such methods had already been employed by Svedberg for the ultracentrifuge, and are also employed in other studies of colloidal solutions such as diffusion measurements. A full chapter in Alexander and Johnson's book [5] is devoted to such methods, and several recent advances are summarised in the papers of Svensson [6].

The geometry of the electrophoretic cell and its construction in sections, as well as the maintenance of the thermostatically controlled optimum temperature (4° C) to

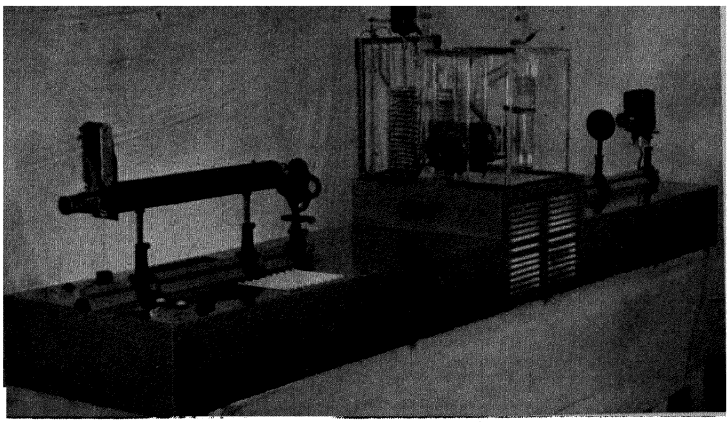

Fig. 3. The Tiselius electrophoresis apparatus constructed by Strübin (Basel).

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prevent diffusion, finally produced a method capable of quantitative analysis of protein mixtures such as serum proteins. A book, entirely devoted to the principles and results of this method, was written by Abramson *et al.* [7].

In Fig. 3 we show the complete apparatus for electrophoretic analysis. This apparatus is extensively used in biological research institutes, but occupies at the moment a place in the laboratory similar to that of the electron microscope. That is, it is under a specialist's supervision

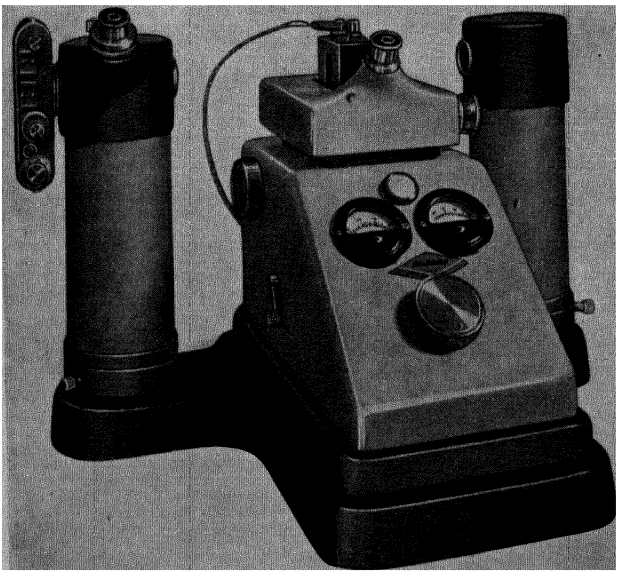

Fig. 4. Antweiler micro-electrophoresis apparatus with Philpot-Svensson schlieren attachment (Shandon Scientific Co., (London).

and requires considerable time and installation both for its construction and manipulation. For general laboratory technique it is too delicate and its cost too high and the organic chemist, as well as the inorganic chemist, is not generally acquainted with its use. A micromethod employing small volumes and a somewhat simpler technique has also been developed (the Antweiler apparatus, Fig. 4).

SCOPE OF THE TISELIUS METHOD

Although electrophoretic separations are possible, usually a "frontal analysis" is carried out. Thus, of a mixture

Fig. 5. Electrophoresis diagram of a normal human serum, by a direct optical method developed by Svensson (obtained with the apparatus of Strübin, Basel).

A–B–C in the central compartment, a small portion of pure A, one of A–B, and another of A–B–C are produced by differential migration, but no separation into bands of pure A, B and C occurs.

Fig. 5 shows a typical derived curve of human serum,

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as obtained by a direct optical method developed by Svensson. Since the Tiselius method has become the basis for electrophoretic analysis, reference will be made frequently to its results, as confirmation of the validity of paper electrophoretic analyses.

The modern techniques of gel and paper electrophoresis have been developed simultaneously with the techniques of column and paper partition chromatography. Several earlier papers described separations using either columns of glass wool, or asbestos fibre, or stacks of filter paper. However, its great scope as a microchemical method seems not to have been realised till a similar micro-technique had been employed in partition chromatography. It was then that paper strips were used, by Wieland and Fischer [8] for amino acids, and by Durrum [9] for proteins, and later by many workers for practically all ionised compounds. A large variety of supports for the paper and apparatuses for the application of the electric field, has since been used.

In a recent review, Tiselius and Flodin [10] pointed out the following advantages of electrophoresis on paper or in porous media over electrophoresis in "free" solution:

"(i) It is possible to obtain complete separation into zones of different migration and thus not only a boundary separation.

(ii) The so-called boundary anomalies interfere less in zone electrophoresis, and therefore substances of low molecular weight (e.g. amino acids, peptides, nucleotides) may also be studied. In addition, zone electrophoresis

(particularly in filter paper strips) requires only minute quantities of material, and can be performed with simple and inexpensive equipment.

These advantages are gained, however, by the sacrifice of greater accuracy of the boundary method (particularly with regard to mobility and isoelectric point determinations). The supporting medium necessary in zone electrophoresis, introduces new factors which may influence the results in a way which is difficult to control."

TERMINOLOGY

The terminology of electromigration methods is not at all clear, nor has a definite name been decided on so far.

Martin and Synge [11] propose that the process concerned with the movement, in an electric field, of relatively small ions should be called *ionophoresis*; the movement of large molecules, *electrophoresis*; the removal of smaller ions from large molecules and particles, *electro dialysis*. To differentiate the "free" methods from those employing packed columns, paper etc., for stabilisation, Tiselius and Flodin [10] employ the term *zone electrophoresis*, to indicate that a thin zone of material is being fractionated into components of different electrical mobilities. For separations employing filter paper, the terms *paper electrophoresis*, *paper ionophoresis* and *ionography* [12], are employed indiscriminately by numerous workers. The word *electrochromatography* is also used as a synonym for zone electrophoresis, and Strain [13] pleads repeatedly for its adoption. For sepa-

ration inside agar jelly some Czech workers employ the word *agarophorese*.

The early methods of electrophoresis inside packed columns have been reviewed by Svensson [14]. Literature surveys of paper electrophoresis have been written by Wunderly [15], Michalec and Hais [16], McDonald [12], Berlingozzi [22], Parker [23], Munier [24] and Lederer [17]. General articles on electrophoretic methods have appeared by Tiselius [4, 18, 19], and two excellent reviews, dealing with the biochemical applications of zone electrophoresis, were published by Tiselius and Flodin [10] and by Kunkel [20]. A valuable source of references, to articles in medical journals, is the literature survey by the paper manufacturers Schleicher and Schüll [21].

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2. Electrical Conduction of Solutions

ESSENTIALS OF ELECTROCHEMISTRY

In a discussion of electrophoretic methods, a recapitulation of some of the essentials of electrochemistry is necessary. We shall briefly recall that the theory of ionic dissociation is based mainly on the evidence supplied by the phenomena of electrolysis, electrical conduction of solutions and the anomalous cryoscopic properties (depression of freezing point, elevation of boiling point) of salts, acids and bases.

The electrical resistance of any material is usually expressed as Ohm's law, which states that the current flowing is proportional to the voltage applied:

$$\frac{E}{I} = R$$

where E is the voltage in volts, I the current in amperes, and R the resistance in ohms. For quantitative measurements one uses the *specific resistance* or *resistivity*, which is defined as the resistance of a piece of material 1 cm long and 1 cm^2 in cross section.

In measurements of electrical conduction of solutions, the reciprocal of the specific resistance, called the conductivity or specific conductance (usual symbol K), is most commonly employed.

Since the conductivity is a property of the ions, the

equivalent conductivity is used to compare the conductivity of different ionised substances under the same conditions. The equivalent conductivity Λ may be expressed as

$$\Lambda = KV$$

where V is the volume containing 1 gram equivalent. Conductivity is usually measured with alternating current, to prevent polarisation of the electrodes and electrolysis. The electrodes used are flat pieces of bright or blackened platinum, and to correct for the geometry of the apparatus (distance between the electrodes and surface area) one measures the cell constant with a solution of known equivalent conductivity e.g. $N/10$ KCl at a known temperature.

Several properties of ions, important for paper electrophoresis, have been observed in conductivity measurements:

TEMPERATURE VARIATION OF CONDUCTIVITY

Table 1 shows the conductivity of $N/10$ KCl between 0°C and 25°C.

TABLE 1

CONDUCTIVITY OF $N/10$ KCl

Temperature, °C	Conductivity
0	0.007129
10	0.009316
18	0.011163
25	0.012852

It will be seen that the increase of conductivity from 0°–25° is almost 50%. This increase is due to the faster movement of the ions in the solution, and must be borne in mind in paper electrophoresis, when considering changes of current during heating of the paper and increased mobilities at higher temperatures.

VARIATION OF CONDUCTIVITY WITH DILUTION

When the specific conductivity of a solution is measured, with progressive dilution of the solution, it is found that it increases to a maximum at "infinite dilution". α is used to express the apparent degree of dissociation where $\alpha = \Lambda_v / \Lambda_\infty$. A typical curve is shown in Fig. 6 and the data for KCl at 18°C in Table 2.

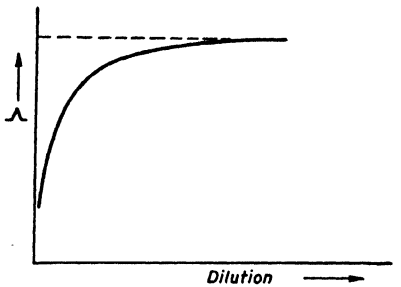

Fig. 6. Conductivity – dilution curve for KCl.

TABLE 2

CONDUCTIVITY OF KCl SOLUTIONS AT 18 °C.

Concentration gm equiv./1	Volume contg. 1 gm equiv. in ml	Specific conductivity	Equivalent conductivity	Apparent degree of dissociation
0			129.9	(1.00)
0.0001	10,000,000	0.00001291	129.1	0.994
0.001	1,000,000	0.0001273	127.5	0.980
0.01	100,000	0.001224	122.4	0.943
0.1	10,000	0.0112	122	0.862
1	1,000	0.0983	98.3	0.757

The important thing to note in this table is, that a completely dissociated salt such as KCl behaves as if only 86 % dissociated in a $N/10$ solution. Most electrophoretic work is carried out near this concentration, and the approximate magnitude of the effect of concentration should be borne in mind.

This apparent lack of complete dissociation can also be observed practically in the same degree in cryoscopic measurements and has been dealt with notably by Debye and Hückel and by Onsager. These workers explain the effects in a concentrated solution by considering the ionic atmosphere i.e. the ions of opposite charge which surround each ion. Any movement of the ions is retarded by an electrical friction effect, which increases as the concentration of the solution rises. Some recent work with ion exchange equilibria shows that another effect, that of actual complex formation, is being given too little attention. The

work on inorganic paper electrophoresis confirms many of these results. (For further details see 18, p. 149). To conclude, the movement of ions in reasonably concentrated solutions is greatly retarded by electrical attraction, owing to their close proximity, and the possibility of the formation of reversible complexes considerably enhanced.

INDEPENDENT MOBILITY AND ABSOLUTE VELOCITY OF IONS

Kohlrausch found that the equivalent conductance at infinite dilution (Λ_∞) of salts consists of two additive factors, which he concluded to be due to the velocities of the cation and the anion of the salt.

For example

				Difference due to difference of velocity of K-Na
$\Lambda_{\infty\,KCl}$	130.1	$\Lambda_{\infty\,NaCl}$	108.99	21.11
$\Lambda_{\infty\,KNO_3}$	126.5	$\Lambda_{\infty\,NaNO_3}$	105.33	21.17
Difference due to difference of velocity of Cl-NO_3	3.6		3.66	

Thus, if the mobility of one ion is obtained, comparative values of the velocities of ions (called ionic mobility) can be obtained from the equivalent conductance measurements at infinite dilution. Some of these values are given in Table 3; it must be kept in mind that the mobilities are only valid at infinite dilution and in the absence of other ions.

TABLE 3

IONIC MOBILITIES

Ion	Mobility	Ion	Mobility	Ion	Mobility	Ion	Mobility
Li^+	33.4	Cs^+	68	$C_2H_3O_2^-$	35	$\frac{1}{2}Cu^{++}$	45.9
Na^+	43.4	H^+	313.9	OH^-	174	$\frac{1}{2}Cd^{++}$	46.4
F^-	46.6	NH_4^+	64.3	NO_3^-	61.8	$\frac{1}{2}Sr^{++}$	51.9
Ag^+	54.3	$\frac{1}{2}Mn^{++}$	28	ClO_4^-	64	$\frac{1}{2}Ba^{++}$	55.4
K^+	64.6	$\frac{1}{2}Co^{++}$	43	$\frac{1}{2}Ni^{++}$	44	$\frac{1}{2}Pb^{++}$	60.8
Cl^-	65.5	SCN^-	56.7	$\frac{1}{2}Fe^{++}$	45	$\frac{1}{2}SO_4^{--}$	68.5
Tl^+	65.9	IO_3^-	34.0	$\frac{1}{3}Fe^{+++}$	61	$\frac{1}{2}CrO_4^{--}$	72
I^-	66.25	ClO_3^-	54.87	$\frac{1}{3}Cr^{+++}$	45	$\frac{1}{2}CO_3^{--}$	60
Br^-	67.7	BrO_3^-	47.6	$\frac{1}{2}Mg^{++}$	45.9	$\frac{1}{4}Fe(CN)_6^{----}$	95
Rb^+	67.5	IO_4^-	48	$\frac{1}{2}Zn^{++}$	47.0	$\frac{1}{3}Al^{+++}$	40

These mobilities may be used to calculate the conductance of fully ionised substances only, thus the conductance

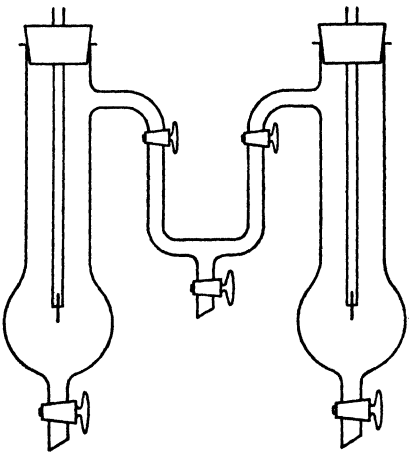

Fig. 7. Apparatus for the determination of Transport Numbers.

of sodium acetate may be arrived at, but not that of the partially ionised acetic acid. It may be noted in this table, that the mobilities of ions of the same charge do not differ greatly, while divalent ions, as may be expected, have twice the mobility of monovalent ions.

When electrolysis is carried out in U tubes with a few taps or constrictions (Fig. 7), it is possible to show that the compositions near the electrodes differ from each other and the centre of the solution. The concentration shift is due to the fact that, with a few exceptions, anion and cation have unequal speeds. This concentration change is measured as the *Transport number* where the

$$\text{Transport number} = \frac{\text{fall in concentration round the cathode (or anode)}}{\text{fall in concentration round the anode and cathode}}$$

That is, the speed of the ion is expressed as the % of the total current carried by both ions.

Salts which have ions of practically equal speeds are KCl, NH_4NO_3, NH_4Cl and KNO_3.

3. Theoretical Considerations

FACTORS INFLUENCING THE MOVEMENT OF PARTICLES IN AN ELECTRIC FIELD

The actual charge of an ionised substance

As mentioned in the previous chapter, the ionic atmosphere has a retarding influence on the movement of ions in an electric field. In addition to this influence (Debye-Hückel effect), we must consider the conditions which will lead to a reduction of complete ionisation.

Metal ions form numerous complexes, which reduce the otherwise relatively high charge on small ionic volumes. It was recently shown that in solutions of HCl, for example, there is no free Fe^{+++}. In a very dilute solution, $FeOH^{++}$ and $Fe(OH)_2^{+}$ appear to be the main forms of trivalent iron, and at higher concentrations of HCl a gradual transition of chloride complexes

$$FeCl^{++} \rightleftarrows FeCl_2^{+} \rightleftarrows FeCl_3^{\circ} \rightleftarrows FeCl_4^{-}$$

has been observed by spectrochemical and equilibrium studies.

As will be seen in the chapter on inorganic separations (**18**), the behaviour of Fe^{III} here mentioned is the rule rather than the exception and, except for alkalies and alkaline earths, practically all ions exist in more or less complexed states.

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Weak acids and weak bases. The pH of the electrolyte used will give a certain amount of ionised and unionised acid in equilibrium. It must be remembered, that this is a completely reversible equilibrium and that at a pH which gives, let us say, 50 % ionisation, the acid will not travel as two bands of ionised and unionised, as would be the case in a non-equilibrium dissociation, but as one band slowed down by the unionised portion. The relation between the dissociation constant K and the pH is given by the Henderson-Hasselbach equation:

$$\mathrm{pH} = \mathrm{p}K + \log \frac{[\text{ionised}]}{[\text{unionised}]}$$

Amino acids with ionisable acidic and basic groups will exhibit cationic and anionic movement, according to the pH of the electrolyte. The pH at which the molecule carries no charge, is known as the *isoelectric point.*

It may be of interest, at this stage, to consider qualitatively the optimum conditions of separation of three similar ions, which have the same mobility when fully ionised. For metal ions a complexing agent, at a concentration or pH yielding the unionised form for the intermediate of the three, would be optimum, as the one which is more strongly complexed will be anionic, and the one less strongly complexed cationic.

For amino acids, the pH at the isoelectric point of the intermediate amino acid would yield cationic movement for the more basic, and anionic movement for the more

acidic ion. From this it will also be apparent that separation of two substances of the same mobility and of the same dissociation constant is theoretically impossible, although differential adsorption on the paper may sometimes produce a separation.

These considerations were elaborated mathematically by Consden, Gordon and Martin [1] for the case of two *weak acids* or other weak electrolytes as follows:

Consider two acids HA and HB with dissociation constants K_a and K_b and ionic mobilities u_a and u_b, then the net mobility of HA (i.e. the movement of a band in an ionophoretic apparatus) will be

$$U_a = \frac{u_a[\mathrm{A}^-]}{[\mathrm{HA}] + [\mathrm{A}^-]} = \frac{u_a K_a}{[\mathrm{H}^+] + K_a}$$

and the difference in net mobility

$$U_a - U_b = \frac{u_a K_a}{[\mathrm{H}^+] + K_a} - \frac{u_b K_b}{[\mathrm{H}^+] + K_b}$$

It can readily be shown that the difference in net mobility is maximum when

$$[\mathrm{H}^+] = \sqrt{(K_a K_b)} \left(\frac{\sqrt{\frac{u_a}{u_b}} - \sqrt{\frac{K_a}{K_b}}}{1 - \sqrt{\frac{u_a K_a}{u_b K_b}}} \right)$$

or

$$\mathrm{pH} = \frac{\mathrm{p}K_a + \mathrm{p}K_b}{2} - \log \left(\frac{\sqrt{\frac{u_a}{u_b}} - \sqrt{\frac{K_a}{K_b}}}{1 - \sqrt{\frac{u_a K_a}{u_b K_b}}} \right)$$

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When $K_a > K_b$ (i.e. $pK_a < pK_b$) and except when K_a/K_b lies between the values u_a/u_b and u_b/u_a and if the optimum pH be used, the maximum difference in net mobility may be shown to be

$$(U_a - U_b) = \frac{u_b\left(\sqrt{\left(\frac{u_a K_a}{u_b K_b}\right)} - 1\right)^2}{\frac{K_a}{K_b} - 1}$$

A chart, showing the relationship of net mobility and ion mobility and dissociation constants, was worked out and applied to the separation of pairs of amino acids by silica gel electrophoresis.

The optimum pH values for the separation of several pairs of amino acids were calculated to be as follows:

glycine – serine	pH 9.2
methionine – alanine	pH 9.4
glutamic and aspartic acids	pH 7
lysine and histidine	pH 6.6
cysteic from aspartic and glutamic acids	pH 6.6.

The charge on a colloidal particle

The charge on colloidal molecules, such as proteins and mucins, originates both from the dissociation of groups, such as COOH and NH_2, and from adsorption of ions in solution. The arguments concerning pH variations for amino acids and the Debye-Hückel effect will thus apply also to colloidal molecules. As however the particles are very large in relation to the ordinary ions, the ionic atmosphere attracted by the surface charges on the colloid will

form a layer of higher ionic concentration of the charge opposite to that of the colloid than in the bulk of the solution. This layer is known as the *electrical double layer*. The random motion of the ions will slightly distend this layer, and for quantitative purposes the diffuse double layer is considered. The colloidal particle, together with its diffuse double layer, forms the electrokinetic unit, and the movement of the colloid will depend on the overall charge of this unit and not on the charges on the surface of the colloidal particle.

The charge on colloidal molecules is usually expressed as the work done in bringing unit charge from infinity to the surface of shear (i.e. the outer surface of the solvated layer surrounding the particle). This is called the *zeta potential*.

Derivation of a simple equation for the speed of one type of colloidal particle

The theory of electrophoretic movement in free solution has been worked out and full accounts, for example, in the book of Abramson *et al.*[2], can be found. A simple equation was derived by Alexander and Johnson[3] for small non-conducting particles, where the diffuse double layer is larger than the radius of the particle. Geometrical factors influence the speed of particles in the electric field and their shape must always be considered.

If the particle possesses a net charge Q, then if X is the field strength, the force producing migration is QX. The

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particle will therefore attain a terminal velocity such that the frictional resistance of the medium in which it moves just balances the electrical force.

If the particle is spherical and Stoke's law can be applied then

$$QX = 6\pi\eta a v \qquad (1)$$

where η = viscosity of the medium
a = radius of the particle
and v = the velocity of migration

Further introducing the mobility u and rearranging we obtain

$$u = \frac{v}{X} = \frac{Q}{6\pi\eta a} \qquad (2)$$

Thus the velocity is governed by the particle size and net charge. Alexander and Johnson also derive equations for a number of other particles, taking geometrical factors into consideration, which however only change the numeral (i.e. the 6 for a 4 or 8 etc.) in the equation (2) above.

Electro-osmosis

If charged colloidal particles are kept fixed in an electric field, for example by the formation of a network such as in a gel or as in a polymer such as cellulose, then the liquid surrounding the gel must move to obtain thermodynamically the same conditions as in electrophoresis. This movement of the electrolyte is called electro-osmosis and can be shown [3] to obey an expression similar to that of equation (2) namely:

$$u_o = \frac{v_o}{X} = \frac{\zeta D}{4\pi\eta} \qquad (3)$$

where u_o is the osmotic mobility, v_o the osmotic velocity, ζ the zeta potential and D the dielectric constant of the medium.

The importance of electro-osmosis in measurements of mobilities in capillary tubes was stressed by Alexander and Johnson [3]. The effect is still greater and of more importance in such media as gels and filter paper.

THEORY OF PAPER ELECTROPHORESIS

Having briefly outlined the factors influencing the movement of particles in an electric field, we shall now present the theory of Kunkel and Tiselius [4] for filter paper electrophoresis. This theory is the most complete so far advanced, and was worked out for an apparatus in which the flow of electrolyte, owing to syphoning and the evaporation of the solvent, is negligible. The problem approached in this theory is the calculation of ionic mobilities from the distances travelled on paper electropherograms.

Correction for electro-osmosis

Kunkel and Tiselius [4], while working on the separation of serum proteins with pH 8.8 barbital buffer of an ionic strength 0.1, noticed electro-osmotic movement due to the negative charge of the filter paper. Filter paper has usually a certain content of COOH groups, which at pH 8.8. would be largely ionised. In order to measure the

electro-osmotic movement, Kunkel and Tiselius placed a spot of dextran on the paper next to the spot of serum protein. Dextran is a neutral polysaccharide which stains with bromophenol blue (like the proteins) and its movement was assumed to follow exactly the electro-osmotic movement. See also Jermyn and Thomas [8].

It was noted that thicker, coarser papers give greater dextran migration than fine, thin paper.

The migration of a specific protein, e.g. albumin, on paper is calculated as

$$-u_{alb} = \frac{d_{alb} + d_{dex}}{Ft} \tag{4}$$

where d is the distance travelled, F the field and t the time. The electro-osmotic flow is calculated as

$$u_{el} = \frac{d_{dex}}{Ft} \tag{5}$$

combining

$$\frac{d_{dex}}{d_{alb} + d_{dex}} = \frac{u_{el}}{-u_{alb}} \tag{6}$$

The ratio of $\frac{u_{el}}{u_{alb}}$ and $\frac{d_{dex}}{d_{alb}}$ should not vary with the distance, or the time, or the electric field, in an apparatus without syphoning of liquid and evaporation, but should vary solely with the charge and porosity of the paper.

This was confirmed with Munktell 20, Ford blotting, and a thick soft paper. The results are in Table 4.

TABLE 4

THE d_{dex}/d_{alb} RATIOS FOR VARIOUS PAPERS

Paper	Ratios calculated from several paper strips			
Munktell 20	0.36,	0.34,	0.35,	0.35
Ford blotting	0.84,	0.70,	0.83	
Thick soft paper	1.7,	1.5		

u_{el} was also calculated for Munktell 20 paper, and $u_{el} \cdot 10^5$ was found to be 1.83, 1.53, 1.69 and 1.61 for four determinations.

Determination of the mobility of ions

We have shown that in free solution the mobility u is

$$u = \frac{v}{X} \tag{7}$$

where v is the velocity and X the field strength.

Expressing this in the quantities usually measured on the paper

$$u = \frac{dl}{tV} \tag{8}$$

where d is the distance, V the voltage, l the length of the tube and t the time, or

$$u = \frac{dqK}{ti} \tag{9}$$

where q is the cross-sectional area, K the conductivity, i the current and t the time.

However, the two above equations cannot be applied to movement inside filter paper, since l the length of the

References p. 30

tube is not the length of the channel inside the network of paper fibres, nor d the actual distance the ions move,

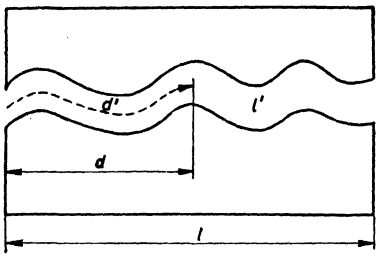

Fig. 8. Movement inside filter paper. The actual distance d' and the channel length l' are not the ones measured on the paper.

but rather a length l' and a distance d' have to be considered as shown schematically in Fig. 8.
Since

$$l' = l\left(\frac{l'}{l}\right), \tag{10}$$

therefore

$$d' = d\left(\frac{l'}{l}\right). \tag{11}$$

A particle in free solution migrates through a distance

$$d = ut\frac{V}{l}, \tag{12}$$

but in paper through a distance d'

$$d' = ut\frac{V}{l}. \tag{13}$$

Substituting (10) and (11):

$$d\frac{l'}{l} = \frac{utV}{l\left(\frac{l'}{l}\right)} \tag{14}$$

and

$$d = ut\frac{V}{l}\left(\frac{l}{l'}\right)^2 \tag{15}$$

Also since in free solution the protein particle migrates a distance

$$d = \frac{uti}{qK}, \tag{16}$$

in paper this becomes

$$d' = \frac{uti}{q_a K} \tag{17}$$

(q_a being the cross sectional area of the paper).
Substituting (11)

$$d\frac{l'}{l} = \frac{uti}{q_a K} \tag{18}$$

and

$$d = \frac{uti}{q_a K}\cdot\left(\frac{l}{l'}\right) \tag{19}$$

That is, equations (15) and (19) differ from the free electrophoresis by the term l/l', the correction factor for calculating the mobility on paper.

It was possible to determine the correction factor using $l' = R\,q_a\,K$ where R is the resistance of the paper strip. Table 5 gives the mobilities for human albumin in barbital pH 8.8, ionic strength 0.1 buffer for various types of paper.

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Values for the correction factor l/l' used in obtaining the true mobility u_c are shown.

TABLE 5

Paper	u_a	l/l'	u_c
Munktell 20	4.01 ± 0.09	0.77 ± 0.03	6.78
Ford blotting	3.21 ± 0.12	0.70 ± 0.04	6.53
S & S 413	2.34 ± 0.21	0.58 ± 0.06	6.85
in the free solution			6.43

The above theory as stated before, is only applicable to one type of apparatus (see page 41); it also does not consider adsorption and other phenomena.

Adsorption on filter paper

The adsorption, especially of proteins, on various filter papers has been examined by numerous methods: Michl [6] applied bands of proteins to various brands of filter paper, eluted them after a certain time and dyed the strips, thus revealing irreversibly-held protein on the paper. He records strongest adsorption with Schleicher and Schüll papers (Nos 598, 595, 602h and 589), much less with Whatman papers (Nos 1 and 4), and least with blotting paper and a Czech paper W.B. 28. Mariani [5] applied large quantities of proteins to the paper, and revealed comets which would have remained invisible with smaller quantities. A summary of the observations of the work of Kunkel and Tiselius [4], and further work by Kunkel [7], describes three other methods: (i) The material is allowed

to move through the paper by liquid flow (i.e. chromatographic development) and the comets thus formed illlustrate the amount of adsorption under the conditions used. (ii) Two-dimensional electrophoresis (page 91) revealed some materials that do not travel as round spots, but as trails, the same buffer being employed in both directions. (iii) Bands of proteins were electrophorised first in one direction and by reversing the current returned to the point of application. Any tracks remaining are due to adsorption on the paper. Kunkel states that, at present, adsorption on the paper remains a serious limitation of the method, particularly in preparative work.

Numerous classes of substances have, however, been shown not to adsorb at all during electromigration on paper. Radioactive tracers of several ions, alkaloids which exhibit sensitive fluorescence, amino acids and peptides, all have been shown to form round spots without any comet effects. Even in the case of proteins, the adsorption does not interfere with quantitative serum protein determinations as standards can be employed. In the examination and analysis of unknown protein mixtures, the possibility of interference due to adsorption should, however, be kept in mind.

Apparent separations are possible on the paper electropherogram, if two substances are moved by the electroosmotic flow and retarded to a different degree by adsorption on the paper.

Whenever this is the case, the degree of separation may

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vary considerably with the potential and the technique used.

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4. Technique of Paper Electrophoresis

In this chapter we shall discuss the essential features of the various techniques employed, giving their main differences, and also some of the commercial apparatus available. We shall limit ourselves to the technique of the actual separations. The methods of qualitative and quantitative analysis employed in conjunction with electrophoresis are mentioned in the sections dealing with the substances separated.

METHODS OF SUPPORTING THE FILTER PAPER

The technique of Wieland and Fischer [1]

Here the paper strip (1 cm × 20 cm) is stretched in a frame made of glass or plastic material (original size 5 cm × 12 cm) with the two ends hanging into beakers containing the electrolyte and electrodes. The paper strip is moistened by dipping it into the electrolyte, the excess liquid is drained off and the sample placed at the centre of the strip, which has previously been marked with pencil.

During the passage of the current, the paper warms up and the electrolyte is evaporated at a rate depending on the current passed. Capillary flow from the electrolyte vessels will make up the loss of liquid due to evaporation. This movement of liquid is not considered in the theory of Kunkel and Tiselius (page 23); it tends to reduce the actual speed of ions on the paper as the flow approaches the centre of the band from both sides.

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In this technique the electrolyte flow does not interfere in the separation of proteins (i.e. at relatively low currents), and Grassmann and Hannig have designed a commercially available apparatus sold under the trade name 'Elphor H'.

A French commercial apparatus based on the same principles and designed by the Centre d'Electrophorèse du C.N.R.S. is sold by Jouan (Paris). This apparatus is shown in Fig. 9.

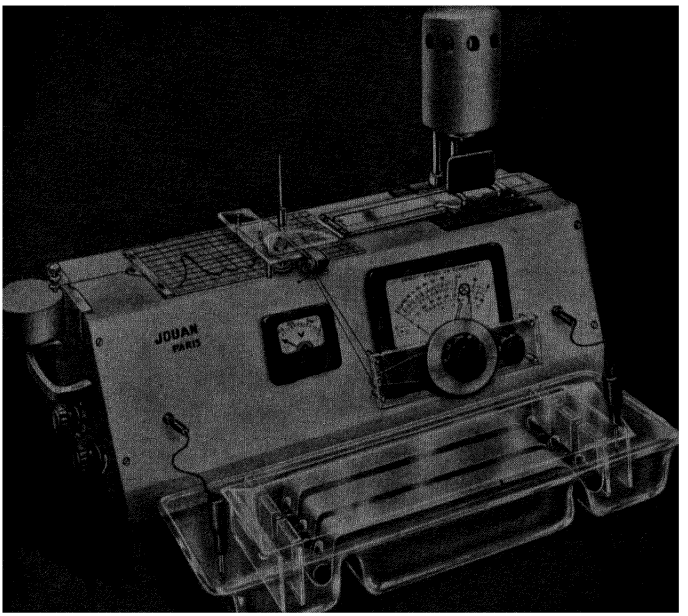

Fig. 9. Horizontal apparatus of the Wieland and Fischer type [1] as constructed by Jouan & Cie. (Paris); photometer with tracing arrangement attached.

The Shandon Co. (England) markets a 'Horizontal Electrophoresis Tank' which can hold three paper strips 5 cm wide (Fig. 10). For an all-glass apparatus see Kawerau[28].

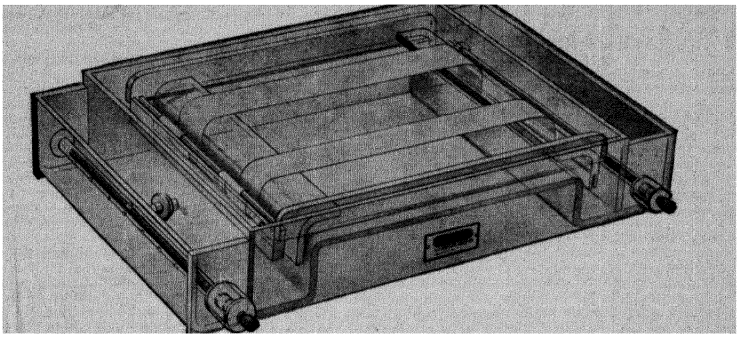

Fig. 10. Horizontal apparatus of the Wieland and Fischer type [1] as constructed by the Shandon Scientific Co. (London).

Another commercially available instrument, called the 'Ionograph' (Precision Scientific Co. U.S.A.), based on similar principles, was developed by McDonald *et al.*[2]. McDonald uses paper strips 90 cm long, which are supported on a frame work, and 'moisture equilibrium vessels' are situated underneath the paper to prevent drying of the atmosphere. 'Feeders' (i.e., paper supports) are used to maintain a uniform water sheath on the paper strips. To ensure rapid cooling, an atmosphere of H_2 or He is used, as these gases are better conductors of heat than air. For most work this apparatus is too complicated and the reader is referred to an excellent review [3] for further details.

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One disadvantage, in techniques employing horizontal paper strips supported only at their ends, is that under certain conditions (usually with thick paper) the paper sags and pools of electrolyte accumulate in the sagged section. The LKB paper electrophoresis apparatus [27] avoids this by resting the paper on a pin cushion support.

The technique of Durrum [4]

Durrum allows the paper to hang over a glass rod at its centre and the two electrode vessels are placed at a lower level than the glass rod. His original apparatus consists of two tumblers covered by a lucite plate, provided with slits for the ends of the paper and the electrodes, and a third tumbler covering the paper support and paper strip. A simple design using a reagent jar as cover, two U-tubes for the electrolyte, and an ordinary stopper as the base was employed by Lederer and Ward [5] as shown in Fig. 11. In later work, Durrum, as well as others [6], used boxes holding a number of paper strips and troughs to hold the electrolyte with horizontally placed carbon electrodes.

The sample to be separated is placed exactly on the centre of the dry paper which is then bent at this spot and hung over the glass support. The electrolyte is then added a little below the apex from a fine pipette. With a little practice one can moisten the sample in this way simultaneously from both sides, and thus concentrate the sample as a fine band exactly at the apex.

Evaporation due to heating, influences the movement of substances to a much greater extent in this method than in the previously mentioned horizontal technique. The flow of electrolyte in a separation run for several hours can be of such magnitude that an 'equilibrium condition' is established; that is, irrespective of the situation of the

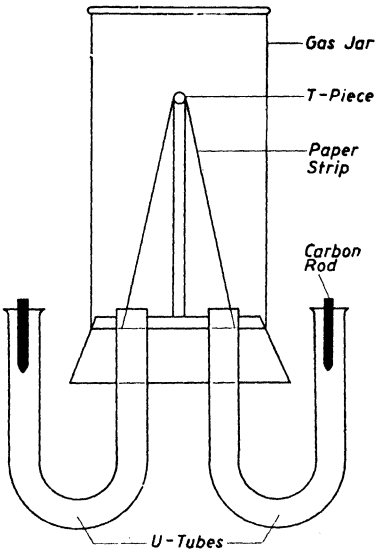

Fig. 11. Simple apparatus after Durrum[4].

original spot (on the apex, or below on either side) and irrespective of the time taken (after a certain minimum) the spots of a certain mobility will be found always in the same place.

It is difficult to visualise that under such conditions satisfactory electrophoretic analyses are possible. However,

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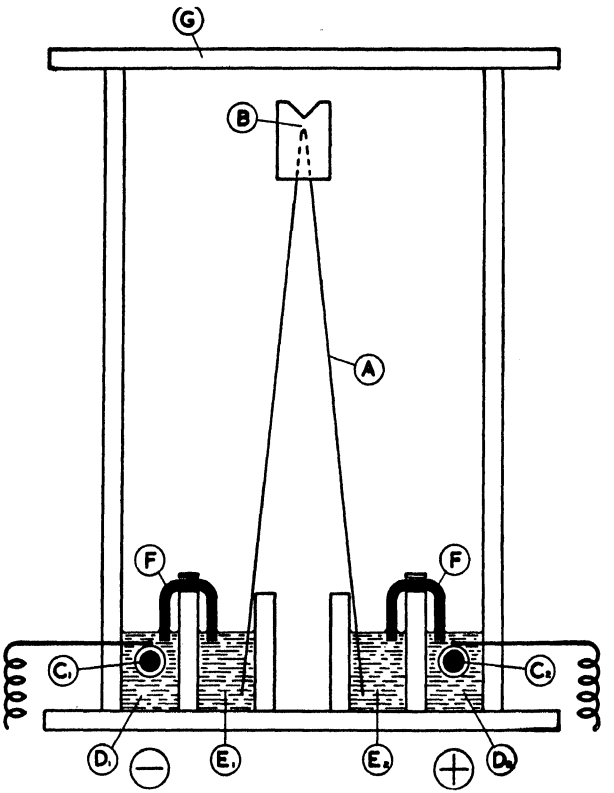

Fig. 12. Apparatus after Durrum [4] constructed by the Shandon Scientific Co. (London) according to a design by Flynn and de Mayo [6].

A filter paper
B suspension wire
C_1 and C_2 carbon electrodes
D_1 and D_2 electrode buffer compartments
E_1 and E_2 buffer compartments for the paper strips
F wicks connecting compartments *D* and *E*
G glass lid

the results obtained with proteins by this technique are identical with those obtained by more complex methods and its simplicity has resulted in its clinical application, often in preference to other methods.

One type of this apparatus is available from the Shandon Co. (England), who employed the design published by Flynn and de Mayo [6] (Fig. 12).

A model with cooling coils on the walls of the cover is also available from this company and is recommended for very high voltages.

A model made of a non-transparent plastic material and using a short paper was designed by Macheboeuf and Rebeyrotte and is produced by Laboratoires Leres (Paris).

Some authors also use paper strips supported by two glass rods, with one rod at a higher level than the other (see Gordon *et al.* [7] and Fig. 13).

The technique of Cremer and Tiselius [8]

An early paper by Cremer and Tiselius suggested an improvement on the two preceding techniques by immersing the paper strip in a bath of a water-insoluble organic liquid, so as to cool the paper efficiently. The paper is wetted with the electrolyte, the sample placed on the centre, and the paper then clamped between two glass plates and immersed in a dish of chlorobenzene; the connections with electrode vessels being maintained with glass tubes filled with paper cuttings wetted with the electrolyte (Fig. 14).

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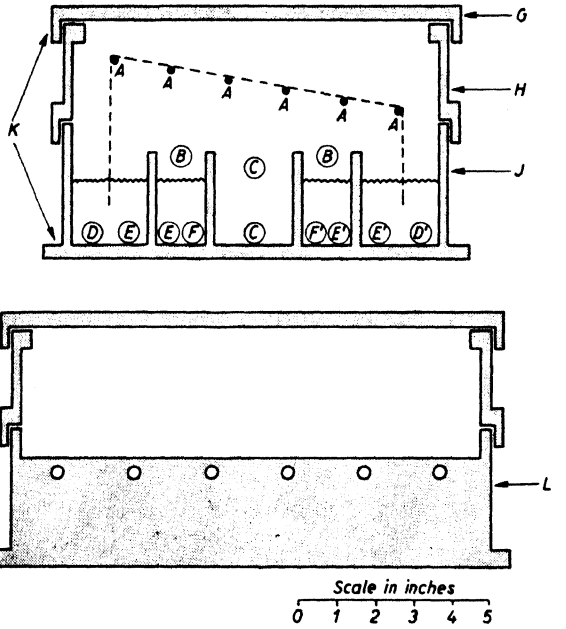

Fig. 13.

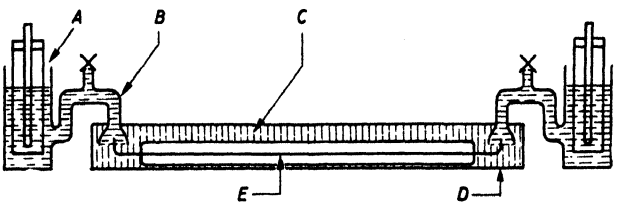

Fig. 14.

Fig. 13. Paper electrophoresis tank after Wolfson [15].

Top: Viewed from one end.
Interrupted line shows position of the paper.
Wavy line: position of buffer level when 2000 ml of buffer are in tank.

A 4 mm glass bars running the entire length of the tank

B female portion of phone jack assembly for plug-in platinum electrode

C water inflow and outlet nipples which permit utilizing the central compartment for cooling purposes: an optional feature chiefly for experimental flexibility

D, *D'* apertures connected by $\frac{1}{4}''$ Tygon tubing to short-circuit the two paper compartments

E, *E'* connected by $\frac{1}{4}''$ Tygon tubing to short-circuit each paper and electrode compartment

F, *F'* connected by $\frac{1}{4}''$ Tygon tubing to short-circuit the electrode compartments

G lid

H paper carrier

J base

K $^3/_8''$ Lucite. All other plastic used is $\frac{1}{4}''$.

Bottom: Longitudinal cross-section of the tank at the level of the barrier separating a paper compartment from an electrode compartment.
The apertures shown permit the passage of wicks 2″ in length: ordinary white shoelaces are entirely adequate.

L buffer level.

(*Note*: It is recommended that the short-circuits *D*, *D'* and *F*, *F'* be placed at one end of the tank, and that the two *E*, *E'* short-circuits be placed at the other end of the tank.)

Fig. 14. Apparatus of Cremer and Tiselius [8] with paper held between glass plates and cooled by chlorobenzene.

A electrode vessels
B U-tubes filled with buffer and paper cuttings
C chlorobenzene
D glass dish
E electropherogram

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This method was somewhat simplified by Schneider [9], but abandoned in a later paper by Kunkel and Tiselius in favour of simply clamping the paper between glass plates, without immersing it in an organic liquid. An apparatus employing a cooling liquid heavier than water (carbon tetrachloride) was used by Smith and Markham [10] (Fig. 15). An arrangement for cooling the paper between

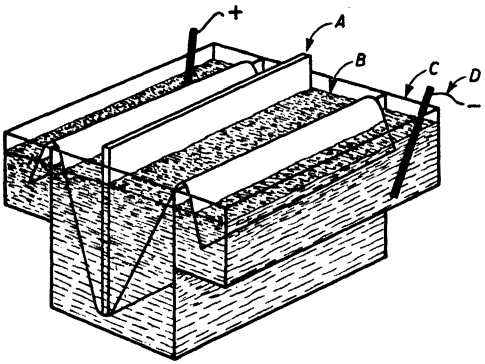

Fig. 15. Apparatus using a liquid heavier than water to cool the paper, after Smith and Markham [10].
A glass plate to hold paper
B carbon tetrachloride
C electrode vessel
D electrode

aluminium plates, which are sealed together with rubber, and covered with a varnish, and cooled on the outside with a water jacket, was described by Michl [11].

The protein separations obtained with some of these techniques were equal to those obtained by the Durrum, and the Kunkel and Tiselius methods.

The technique of Kunkel and Tiselius

It has been shown by many workers [5], [12], etc. that sandwiching the paper between glass or plastic plates may be used as a simple method for cooling the paper. The author has employed the simple apparatus shown in Fig. 16 for solutions of strong electrolytes, where evaporation was too great by the Durrum method.

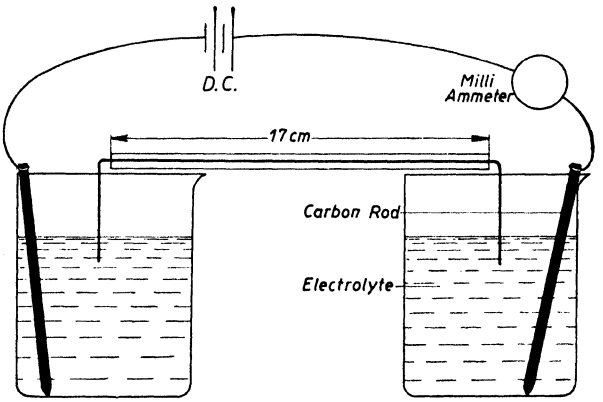

Fig. 16. Simple glass plate apparatus.

However, in some separations with weak electrolytes, glass plates not covered with vaseline or silicone were noted to produce an uneven electro-osmotic flow, which in some cases prevented separation into distinct bands.

Kunkel and Tiselius [13] designed an apparatus in which the paper is held between glass plates, thus reducing all interfering factors to such an extent as to permit the mobility measurements discussed in Chapter 3, p. 23.

The paper is clamped between glass plates (9 cm × 25 cm), and the sides are sealed with silicone grease to prevent evaporation. Electrode vessels of the type shown in Fig. 17 are of such a size as to remain at a constant pH during electrophoresis. The paper touches vertical pieces of very porous paper, which then dip into the electrode vessels. The quantity of serum used for analysis is 0.01–0.04 ml per spot. Using a stack of papers, and cutting a U-shaped tongue into them, up to 1 ml of serum could be separated in one run.

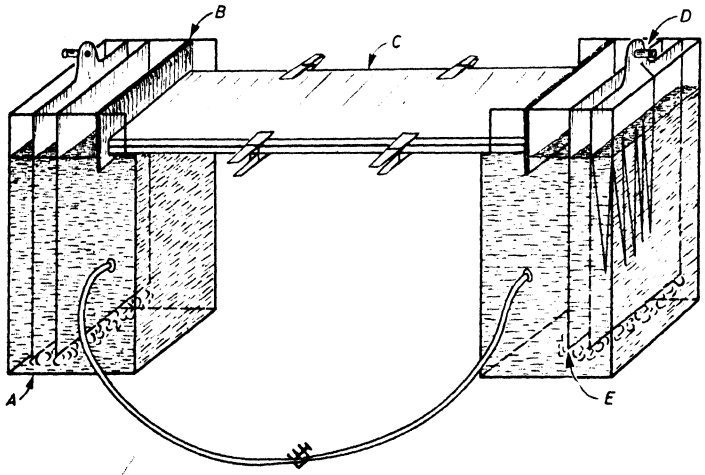

Fig. 17. Apparatus of Kunkel and Tiselius [18].

A perspex electrode vessels
B porous paper for establishing contact between paper and electrode vessel
C glass plates holding the paper
D electrodes
E baffle plate to avoid pH changes near paper

Kunkel [14] reports an improvement to this method. He was able to reproduce the speeds of migration more accurately by sandwiching the paper between glass plates 2.5 cm thick (25 cm × 23 cm) which distribute evenly a pressure of about 1 pound per square inch, applied by means of a special clamp. With such a pressure a uniformly fine film of liquid can be maintained between the glass plates and 10–14 specimens, run parallel, all move exactly the same distance.

Techniques for migration over long distances

One method evolved by Wolfson [15] for the separation of abnormal haemoglobins, which have only small differences in mobility, is as follows. Using the apparatus shown in Fig. 13, 'the end of the paper at the anode is a roll of paper which can be unwound. The cathodic end is the usual strip end. The run is begun with the samples as close to the cathode as possible. When they have reached their equilibrium position (see page 35) the strip is shifted to bring the spots back towards the cathode, the roll of paper is unwound sufficiently to make this possible, and the run is resumed. When the samples get back to the equilibrium position, this is repeated. We have had some runs of twice the distance between the electrodes. Using this technique, it is always possible to differentiate Hgb-A (normal adult) and Hgb-S (homozygous sickle-cell anaemia) when run side by side. In the heterozygous carrier of sickle-cell trait, who has both of the above, we may get two definite bands

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or we may simply have a broad band covering both the Hgb-A and Hgb-S areas. However, in the recently described type III individual who has Hgb-A and another abnormal Hgb-C, we get very striking and excellent separations. All of our studies, unlike those of Linus Pauling, were carried out with native haemoglobins rather than the carbon monoxide derivative'.

In studies of alkaloids of the curare group, Marini-Bettolo and Lederer [16] used a Wieland and Fischer apparatus over 40 cm long. In this apparatus the heavy papers (e.g. Whatman 31 extra thick) sag too much, but thin papers such as Whatman No. 1 may be successfully employed.

The techniques mentioned above will give the reader the idea that numerous variations of these techniques could also be used. This is quite so, and we have omitted a large number of other techniques (for example [17]) which may be equally well applied. At the moment there is no method by which one can choose the best technique in a certain problem except by trial and error. Kunkel and Tiselius [13] state: 'Each type of protein required special conditions because of variations in concentration, solubility in various buffers, mobility, adsorption and numerous other factors.'

High voltage paper electrophoresis

It is evident that the technique of migration over long distances will lose some of its efficiency owing to the increase of diffusion during the longer time required.

The obvious alternative for exploiting small differences in mobility is the use of very high potentials. The first apparatus used for potentials up to 50 volts/cm developed by Michl [17], is merely a variation of the Cremer and Tiselius apparatus using liquid cooling. It appeared, however, that considerable technical improvements would be necessary if high potentials were used with techniques in which the paper is not surrounded by a non-polar liquid, or if even higher potentials such as 100–160 volts/cm were used. Kickhöfen has recently reviewed the work on high voltage electrophoresis [29].

An apparatus capable of using high potentials was constructed by Westphal and Werner [30] and is being manufactured by Dr. Virus, KG, Bonn, Germany.

The apparatus requires a brine-cooled surface whose temperature is so regulated that the paper itself has a constant temperature of −15 to 0°C during electrophoresis. The paper strip (either 60 cm or 100 cm long, depending on the apparatus) is moistened with buffer and then squeezed out in a roller press since excess surface liquid may distort the separations and the cooling effects considerably. During electrophoresis a glass plate (as shown in Fig. 18) is clamped onto the paper lying on the cooling surface so as to leave a moist chamber above the paper. It is essential that any evaporation due to the resistance of the paper should be stopped and a condensation gradient through the paper towards the cooling surface maintained, if necessary with the aid of a heating lamp

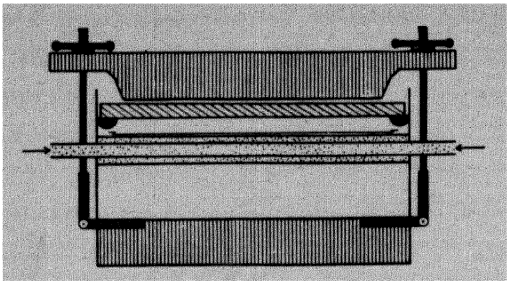

Fig. 18. Cross section through electrophoresis chamber. The two arrows indicate the space for the circulating cooling liquid, above which lies the paper, held in position by two foam-rubber bands (one on each side) and covered with a glass plate. (Werner and Westphal [80]).

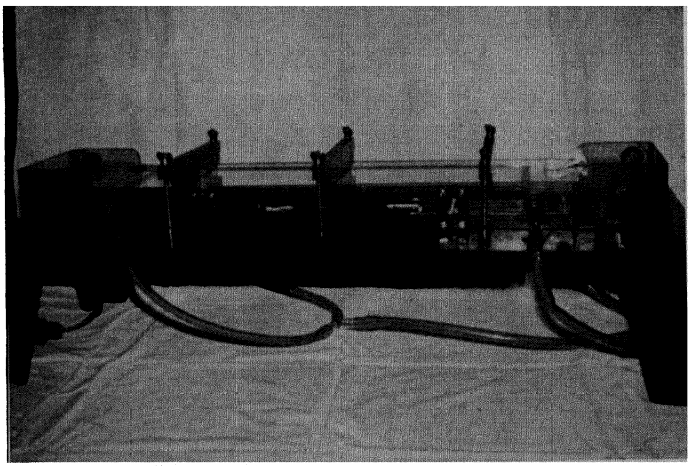

Fig. 19. Apparatus for electrophoresis with high potentials, constructed by Dr. Virus KG, Bonn (Germany).

References p. 53–54

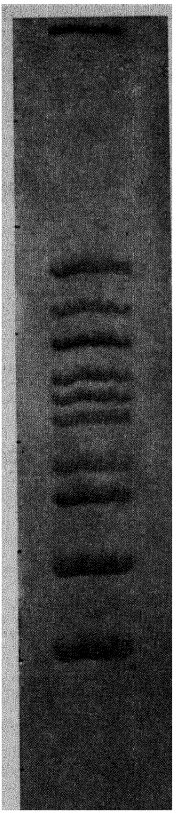

Fig. 20

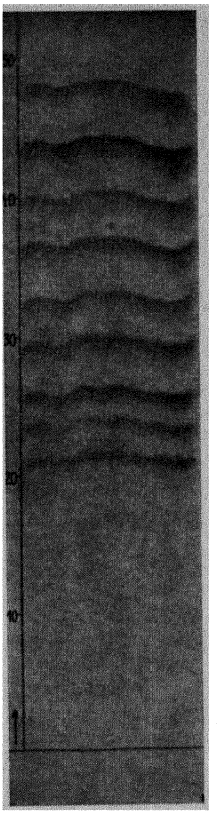

Fig. 21

Fig. 20. Separation of a mixture of amino acids. The bands from top to bottom are: Glycine, alanine, α-aminobutyric acid, serine, threonine, methionine, glutamic acid, aspartic acid, hydroxyproline, dihydroxyphenylalanine, taurine.
pH approx. 2, 60 volt/cm, 1.1 mA/cm band width, cooling liquid at -7° C, time 200 min. (Werner and Westphal [30])

Fig. 21. Separation of a mixture of peptides. The bands from top to bottom are: Glycyl-glycine, alanyl-glycine, triglycine, glycyl-leucine, glycyl-valyl-alanine, glycyl-tyrosine, leucyl-tyrosine, tyrosine, glutathione.
pH approx. 2, 60 volt/cm, 1.1 mA/cm band width, cooling liquid at -11° C, time 210 min. (Werner and Westphal [30])

on the glass plate. Connection of the paper strip with electrolyte vessels is maintained via cellophane membranes to avoid movement of liquid into the rather poorly wetted paper (ca. 110% liquid in the paper).

Fig. 19 shows the assembled apparatus. Although in this instrument no success was obtained with high-molecular substances, excellent results are possible with *small molecules* as shown in Figs. 20 and 21.

Separation with a potential gradient

Michl [31] pointed out that separation conditions as favorable as those sought in chromatography when carrying out gradient elution may be easily obtained in paper electrophoresis by giving the paper a trapezoidal form and allowing a mixture to move from the wider end to the narrow one. Not only will a better separation be obtained in a shorter time but larger quantities may be placed on the paper without overloading since only the initial zone holds the mixture and this is situated on the widest part of the paper.

Radial development

Although radial development, with the sample placed in the centre of the arc as in chromatography, acts against the potential gradient, it has found several adherents. Cetini [32] employs it for the separation of inorganic ions and Berlingozzi *et al.* [33, 34] for proteins.

OTHER TECHNICAL ASPECTS

The solution to be analysed

The effect of large amounts of electrolytes present in the solution to be analysed can be best discussed with an inorganic example: The separation of Bi^{+++} and Cu^{++}, with HCl as the electrolyte on the paper, depends on the formation of negative $BiCl_4^-$; Cu^{++} remaining cationic. If the solution containing Bi^{+++} and Cu^{++} which is to be placed on the paper, contains a high concentration of anions other than Cl^- (e.g. HNO_3), this will prevent the formation of anionic $BiCl_4^-$ till the solution has adequately diffused into the electrolyte surrounding it. As this might require some time, Bi^{+++} will be found to travel first to the cathode and later to the anode. Thus, the speed of Bi^{+++} and the degree of separation of Bi^{+++} from Cu^{++} will be less than in a solution which is isotonic with the electrolyte and contains no foreign anions.

This effect was observed with proteins (called *Umkehreffekt*) by Graf and List [18], who noted that whole horse-serum gave different patterns when the time of running the electrophoretic separation was varied, and that the order of bands changes during 20 hours of electrophoresis. The same serum when first dialysed against the buffer used showed linear movement with respect to time. In this case, the movement of the serum fractions is influenced by the blood electrolytes, whose presence changes the charge of the colloidal ions.

References p. 53–54

Dialysis is not usually used in the analysis of human serum, and as long as standard conditions are employed comparable results will be obtained. This effect may however account for small differences in the patterns obtained by various methods.

Electrodes

In practically all methods either carbon or platinum electrodes are used. The latter must be of sufficient area to prevent undue polarisation. Carbon electrodes dipping into electrode vessels are best connected with platinum wires to the source of current to prevent the introduction of impurities from attackable wires.

The source of direct current

The usual source of direct current is a rectifier delivering between 100 and 300 volt. For some separations and for the continuous method (see page 55) a higher potential is used, and a source of up to 1000 volt has frequently been employed. See also Tsao [26]. For most separations a series of 45 volt dry batteries may be used equally well and will yield a constant potential for several months.

An ordinary milliammeter is usually included in the circuit to indicate that the current passes and that all contacts work satisfactorily. A voltmeter with two Pt wires as terminals can be used to measure the voltage drop along the paper directly, by touching the paper strip, with a known distance between the wires.

PREPARATIVE METHODS

Most of the techniques described on pages 31–44 lend themselves to the separation of quantities up to a few hundred milligrams, by employing either a stack of filter papers [13] or a single thick paper strip.

Of the numerous examples published in the literature we shall quote the paper by Hartmann and Müller [19] who apply the apparatus of Wieland and Fischer [1] with a 2 mm thick paper (Schleicher and Schüll 2071). Using a pH 8.4 buffer and running the separation with 110 volt for 24 hours they separate 200–300 mg of proteins.

Wunderly [20, 21] similarly adapts the Durrum method [4] with extra thick Munktell No. 20/350 paper and separates 0.4 ml of serum on a strip 58 cm × 7 cm. Kunkel and Slater [22], using stacks of Whatman 3 MM (3–9 sheets), separate 3–5 ml of serum in one run. Inorganic separations on strips of paper pulp were recorded by Anderson and Lederer [23], who isolated milligram amounts of Cu from a mixture of Cu with some platinum group metals.

The 'Umkehreffekt' due to extraneous substances present in the solution to be analysed, can be of much greater importance in this technique, as the amount of the solution in relation to the amount of electrolyte is greater. In some preliminary experiments with this technique, the author was unable to separate certain rare earth mixtures which separated readily both in micro amounts and by the continuous method.

References p. 53–54

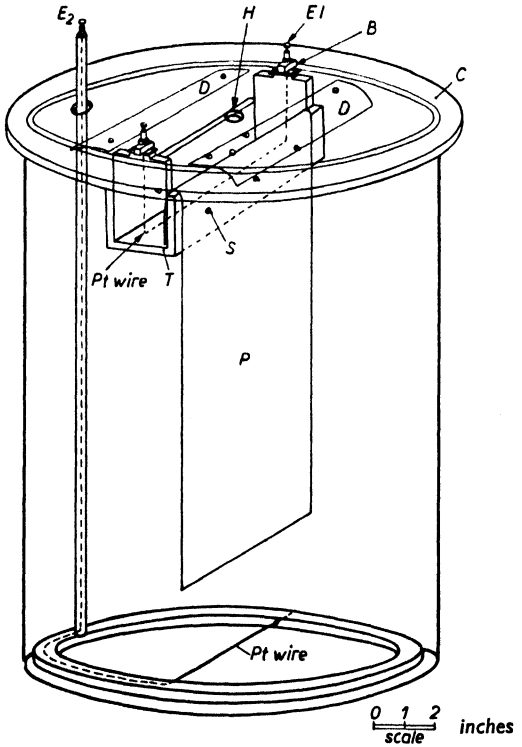

Fig. 22. Apparatus for preparative paper electrophoresis (Cannon and Gilson [24]).

One type of preparative apparatus, based on the method of Michl [17], was employed by Cannon and Gilson [24] in the preparative scale separation of hydrolysis products of Vitamin B_{12} using 0.1 KCN as electrolyte. The amounts of pigments separated were approximately 100 mg. The apparatus is shown in Fig. 22. This method, as well as a preparative method based on the Durrum apparatus, has

been used extensively in the work of Todd *et al.* [25] on the chemistry of vitamin B_{12}.

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5. Continuous Electrophoretic Separation

PRINCIPLE

The principle of applying an electric field at right angles to a flowing liquid inside a porous medium, with a central stream of a mixture to be separated was first proposed by Svensson and Brattsten [1]

The process is depicted schematically in Fig. 23.

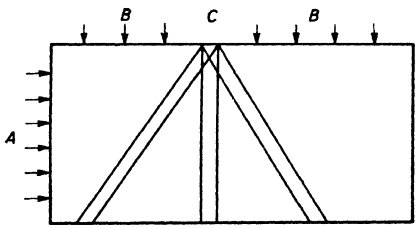

Fig. 23. The principle of continuous electrophoretic separation.

A electric field
B buffer
C sample

The mixture to be analysed and the buffer are flowing at a constant rate through a capillary system, and the electric current is applied perpendicularly to the direction of flow. A charged particle in solution will then migrate in a direction that makes a certain angle with the direction of flow.

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The tangent of the angle is equal to the ratio between the velocities due to the electric migration and liquid flow. If the mixture to be fractionated is injected within a narrow region of the system, the different components will form separate bands in the system with different angles of inclination. With a constant speed of flow and with a constant current, the angles of the different bands will keep constant during the separation. The speed of flow and the current appear to be the main factors which determine the sharpness of separation.

If the difference in mobility between two components in the entering mixture is Δu cm^2 volt^{-1}sec^{-1}, the difference in volume velocity in the direction of the current between the two bands in the column will be

$$V_{el} = \frac{\Delta u \,.\, i}{K} \text{ cm}^3 \text{ sec}^{-1}$$

where i is the current and K the conductivity. If complete separation is wanted, the volume velocity of flow in the perpendicular direction must not exceed this value

$$V_{\text{flow max.}} = V_{el}$$

Δu and K are related to constants of the apparatus only in so far as the presence of a capillary system influences the mobilities (i.e. adsorption), whereas the current is directly dependent upon the design of the apparatus

APPARATUS

The apparatus of Svensson and Brattsten [1]

In the first apparatus of this kind, Svensson and Brattsten use a bed of glass powder in a lucite frame as shown

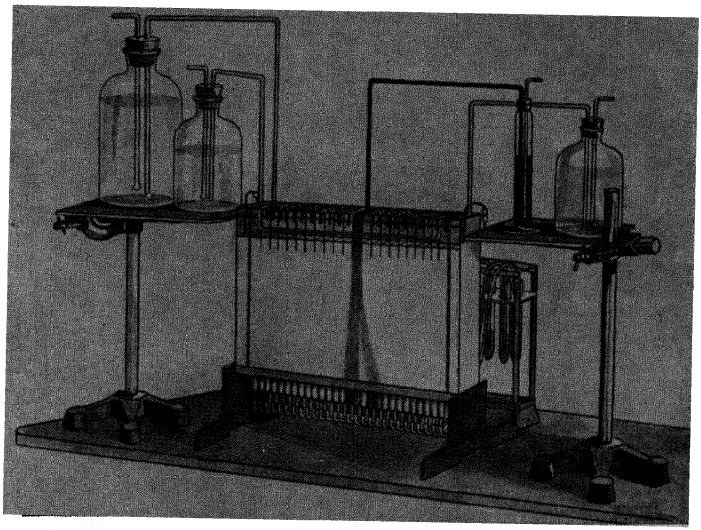

Fig. 24. Apparatus for continuous electrophoresis after Svensson and Brattsten [1].

in Fig. 24. The electrolyte level is maintained and the sample added by means of Mariotte flasks; separate alkali and acid solutions are used for the electrode compartments to avoid pH changes due to electrolysis.

They separated mixtures of dyes in preliminary experiments and the authors consider improvements necessary

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to the design, such as current stabilisation and thermostatic regulation. Mould and Synge [13] separated several starch-iodine complexes in an apparatus which embodies these improvements. See also Brattsten [14].

The apparatus of Grassmann and Hannig [2]

In the publication of Svensson and Brattsten [1] quoted above, the use of filter paper in experiments by Durrum is already discussed. The first successful instrument using filter paper sheets was described, however, by Grassmann and Hannig [2]. The principle of the Svensson-Brattsten apparatus is strictly adhered to. Electrodes are clamped at the sides of the paper sheet which is cut in zig-zag at its lower end with each tongue delivering a fraction into a recipient.

A commercial model is available from the firm Bender and Hobein (Munich, Germany) sold under the name 'Elphor V'. (Fig. 25). Three positions are allowed for the addition of the sample, at the centre or near each electrode. Accounts of very satisfactory separations of human serum proteins and of amino acids and dyes, have been published, by Grassmann [3], and in commercial pamphlets (by Bender and Hobein).

Pfeil and Kanngiesser [8] recently suggested an improved electrode arrangement, to avoid diffusion of electrolysis products from the electrodes into the paper.

The apparatus of Strain [4]

Strain *et al.* [4, 5] used relatively thick paper (3 mm thick)

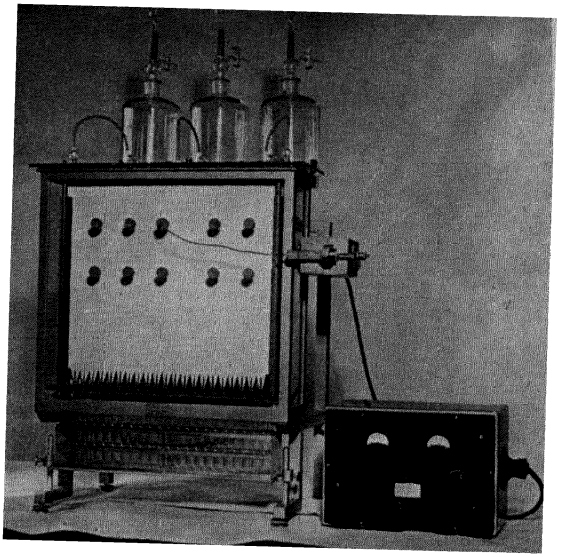

Fig. 25. The apparatus of Grassmann and Hannig - as constructed by Bender and Hobein (Munich). The model shown is the new Elphor V_a apparatus designed to give 48 fractions.

sandwiched between glass plates, for electrophoretic separations of inorganic compounds.

For continuous operations, glass plates 12 × 12 inch and 22 × 24 inch and 0.376 inches thick were used. The sides of the paper were paraffined and platinum electrodes clamped in tightly with the paper between the glass plates. A space is left on the upper part between the glass plates for addition of the electrolyte, and a paraffined paper compartment with a central wick serves for the addition of the sample.

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At the lower end, paper strips are attached to the sheet which dip into recipients such as test tubes. The whole apparatus is schematically shown in Fig. 26. It has the advantage, over instruments with free paper sheets, of having no evaporation and fast cooling, thus being suitable

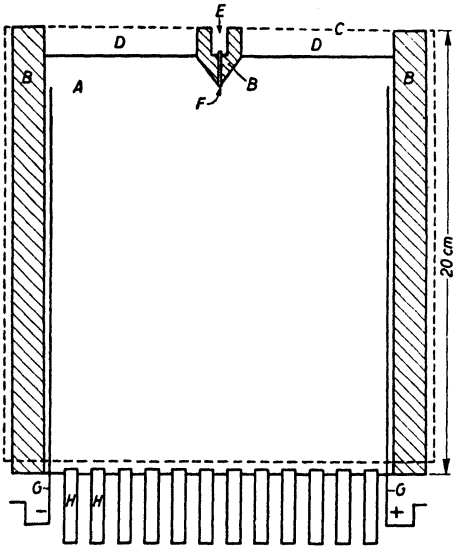


Fig. 26. Electrographic cell for continuous operation (Strain and Sullivan [4]).

A filter paper
B regions of paper impregnated with paraffin
C outline of glass plates
D compartments for addition of wash liquid
E compartment for addition of solute mixture
F paper wick through paraffined region
G platinum electrodes in grooves in glass plate
H paper strips to promote uniform flow of wash liquid

for high currents. All instruments with electrodes the length of the sides suffer from the diffusion of electrode products into the region of the paper used for separation. Thus Strain and Sullivan [5] point out that the use of ammonium acetate as electrolyte produces an acid and an alkaline region as shown in Fig. 27.

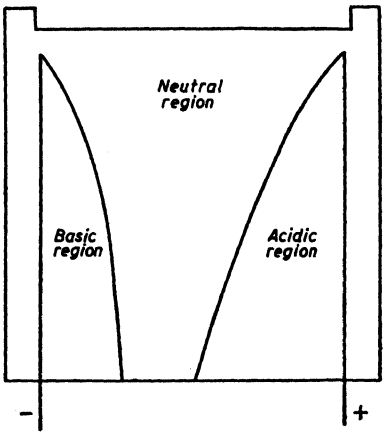


Fig. 27. Acidic and basic regions formed in electrographic cell with 0.1 *M* ammonium citrate. 300 volts, ca. 50 mA.

The numerous inorganic separations achieved with this instrument will be found in **18** (p. 149).

The apparatus of Durrum [6]

This apparatus uses electrodes at the bottom of the paper instead of along the edges, thus producing a curved electric field instead of a linear one, as in the case of the

other instruments described. This is illustrated schematically in Fig. 28. The curved field produces migration in curved paths instead of the linear movement in the linear field. However, the practical advantages are considerable. Instead of closely adhering electrodes, the paper simply dips into beakers and the electrode products have to

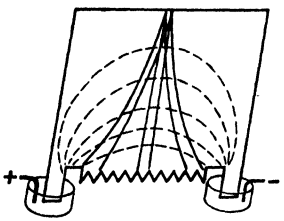
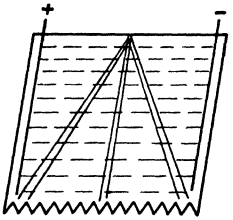


Fig. 28. Curved field (method of Durrum)



Linear field (method of Grassmann and others).

migrate against the stream instead of being produced along the paper; thus they are eliminated or greatly reduced.

The Durrum apparatus is being constructed by the Shandon Scientific Co. (England) (Fig. 29) and by Jouan (Paris).

For other designs see Mondovi and Antonini [15], Saroff [16] and the apparatus of Karler [17].

SOME APPLICATIONS

It was suggested by Grassmann and Hübner [7] that loose addition compounds could be demonstrated by allowing two streams of different substances to intersect each other on the paper.

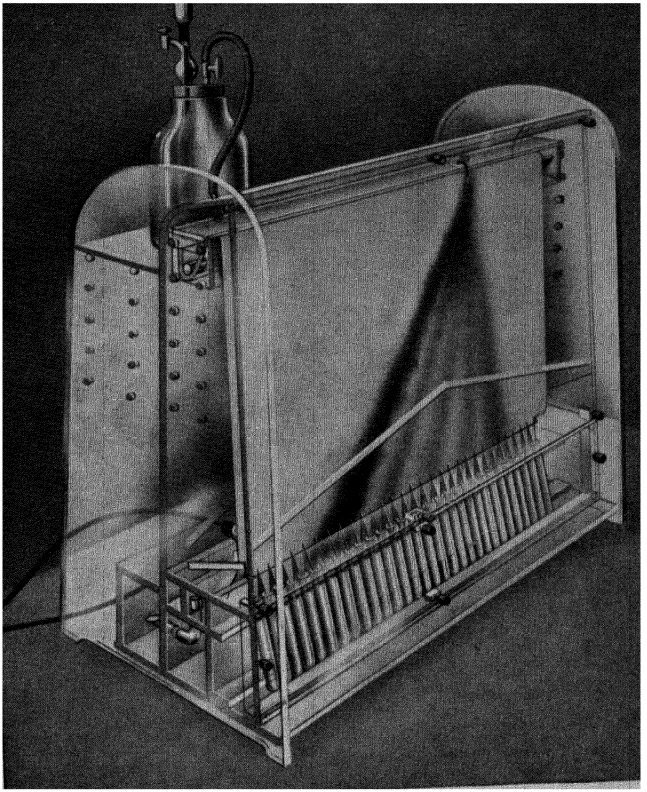


Fig. 29. Continuous electrophoresis apparatus after Durrum [6] as constructed by the Shandon Scientific Co. (London).

When an addition compound is formed, a disturbance of the paths will be seen at the intersection. This was illustrated with Orange II and methylene blue, which exhibited the formation of an addition compound (in *N* acetic acid

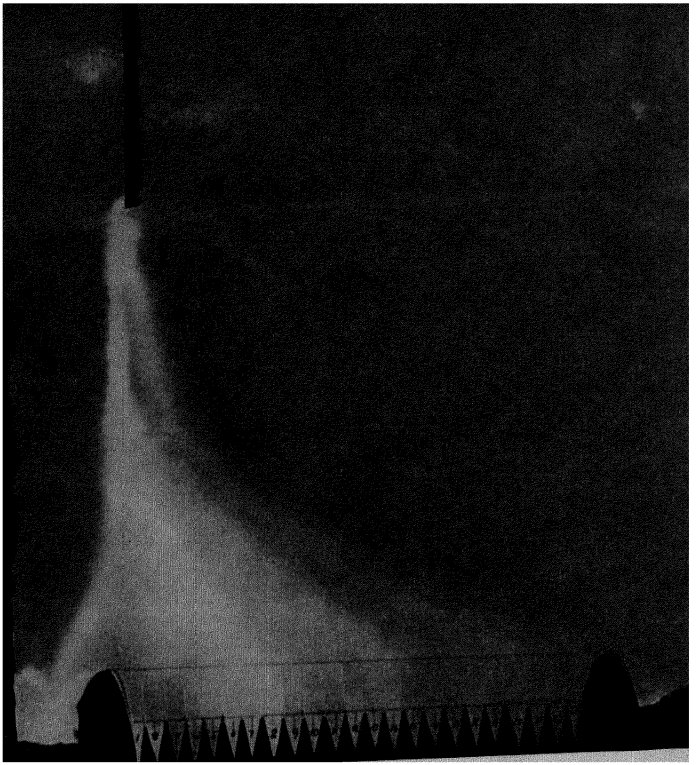


Fig. 30. Continuous electrophoretic separation with the apparatus of Durrum [6]. The ultra-violet fluorescing alkaloids from *Strychnos froesii* are shown. Conditions: 25 % acetic acid, 300 volt, Whatman No. 1 paper.

with 110 volt), while amido black and fuchsin crossed without hindering each other. Some of the possibilities of continuous electrophoresis as a preparative method are shown in the paper by Holdsworth [9]. Using the Durrum technique, he separated 2.5 grams of freeze-dried sow's-

whey proteins in five days. Coenzyme A, flavin adenine dinucleotide and the vitamin B_{12} binding substance were also purified in this apparatus and freed from all protein impurities. Fractionation of human serum albumin was effected by Larson and Feinberg [12].

Amino acid separations were described by Grassmann *et al.* [18].

A not too successful separation of sugars in a borate buffer was recorded by Micheel and Van de Kamp [10]. See also the chapters on alkaloids, amino acids etc. Fig. 30 is a separation of the curare alkaloids from *S. froesii* using the Durrum apparatus, with 25% acetic acid as electrolyte [11].

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6. Electrophoresis on the Cytoscale

A cyto-method capable of quantitative and qualitative analysis of the nucleotides present in ultramicro quantities was described by Edström [1-3].

A piece of copper silk is treated with alkali in order to produce swelling of its fibres. It is washed with water and immersed in a highly viscous citrate buffer of pH 3.6 containing glycerol and glucose. This buffer neither takes up nor gives off water at ordinary room temperatures and humidity, and is of high electrolytic strength. Its viscosity is 1,650 times greater than that of water. After at least 24 hours in this buffer, the silk is taken out and the excess moisture removed by centrifugation. A fibre 1–2 cm in length is stretched out on a quartz slide. Small dabs of paste containing citrate buffer are smeared on both its ends. The quartz slide is then inverted over a groove in a thick glass slide, with the fibre perpendicular to the groove. Hydrolysed ribonucleic acid (1*N* HCl for 1 hour at 100° C), from which the HCl has been removed, is placed at some point along the fibre with a micropipette directed by a micromanipulator. It is applied slowly and is concentrated through the evaporation of the solvent. The work is controlled microscopically. When a suitable quantity (100–1000 pg*) of ribonucleic acid has been ap-

* 1 pg = 1 picogram = 10^{-12} g.

plied, the chamber formed by the quartz slide and the groove is filled with liquid paraffin. Platinum microelectrodes are then inserted into the dabs of buffer paste.

A direct current voltage of 12 v/mm applied for two hours separated four ultraviolet-absorbing constituents of ribonucleic acid, when the buffer had a viscosity of 1,650 centipoises. Less than 1 mm of the length of the fibre is required and several analyses can therefore be made simultaneously. The quartz slide is then removed from the oil chamber and provided with a quartz cover-glass. Liquid paraffin is used as the mounting medium. The fibre is photographed with a light calibrating system in monochromatic light of 257 and 275 mμ wave length respectively. The plates are investigated photometrically and the absolute amount of each base and nucleotide can be computed.

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7. Combination of Chromatography and Electrophoresis

The experiments of Strain [1] and of Lecoq *et al.*, [2, 3] on the application of an electric field to a chromatographic adsorption column, have already been reviewed elsewhere [4].

Numerous authors have found advantages in combining paper partition chromatography with electrophoresis, carried out either simultaneously or separately. We shall only indicate the variants of these techniques in this chapter, as no purpose would be served in enumerating all separations recorded.

SIMULTANEOUS CHROMATOGRAPHY AND ELECTROPHORESIS

Haugaard and Kroner [5] developed this method for the separation of amino acids. Paper sheets were impregnated with phosphate buffers, and copper electrodes attached on the sides of a sheet chromatogram which was developed with saturated phenol, and the potential applied at the same time. Thus a two-dimensional separation was obtained, the amino acids moving, according to their partition coefficients in the direction of the liquid flow, and according to their electrical mobilities in the direction of the electric field at right angles to the solvent flow.

A technical advance was made by Burma [6], who eliminated the electrodes, which are difficult to attach and pro-

duce Cu^{++} spots on the paper, by painting colloidal graphite 2 cm wide on each side of the paper and connecting these 'graphite electrodes' to the source of current. Burma also employs phenol/buffer as solvent and impregnates the paper with buffers before chromatographing.

The simultaneous technique limits the electrophoretic as well as the chromatographic separation, as both are naturally carried out at the same pH.

ELECTROPHORESIS FOLLOWED BY CHROMATOGRAPHY

When electrophoresis and chromatography at different pH values are desired, it is necessary to perform one separation after the other. Among the many possibilities of carrying out these two steps we shall suggest the following:

(i) A paper electropherogram is carried out with a volatile electrolyte, such as acetic acid or a buffer prepared from acetic acid and pyridine. The paper is then dried to volatilise the electrolyte and stitched to a square sheet of filter paper and developed with the desired solvent. This method developed by Marini-Bettolo [7] was used for the separation of curare alkaloids. An example is given in Fig. 30.

(ii) When sufficient material is available a continuous electrophoretic separation is carried out and each fraction obtained evaporated and the electrolyte removed by suitable methods. Any macro-separation on thick paper,

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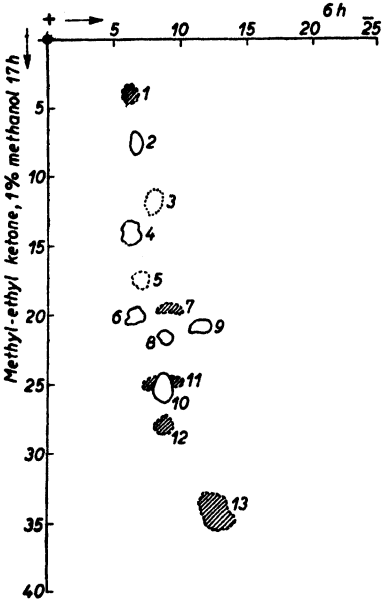

Fig. 31. Electrophoresis followed by chromatography: a separation of the alkaloids of *Strychnos tomentosa*. Electrophoresis with 25 % acetic acid followed by chromatography at right angles with methyl ethyl ketone containing 1 % methanol (Marini-Bettolo *et al.* [7]).

columns or gel can of course be used equally well for the macro-separation. The fractions may then be chromatographed in two dimensions or electrophorised in one and chromatographed in another. Thus three separatory effects may be employed for the characterisation of a mixture.

Recently a scheme of ionophoresis followed by chromatography was worked out by Honegger [8].

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8. Organic Acids

According to the theory of Consden, Gordon and Martin (page 19), organic acids may be readily separated if they have different pK values.

HIGHER FATTY ACIDS

Two methods for the electrophoretic separation of higher fatty acids have been described. In both, retardation by adsorption appears to be the main factor for the differences in mobility.

Barnett *et al.* [1] performed what they term 'string electrophoresis' with a paraffin-impregnated candle wick. Using 3N ammonia and 300 volt, they were able to separate C_{18}, C_{16}, C_{14} and C_{12} acids. For details the original paper should be consulted. Later, Barnett and Smith [2] found that separation of higher fatty acids could also be achieved on filter paper strips. Using a gradient of 6 volt/cm and aqueous ammonia as electrolyte, the C_{10}, C_{12}, C_{14}, C_{16} and C_{18} acids moved 4.5, 1.5, 0.33, 0.07 and 0.0017 cm/hour. The spots were revealed with methyl red/bromothymol blue indicator (sensitivity about 80 μg of C_{16}) No separation of unsaturated and saturated acids of the same chain length could be effected. It was possible to separate lauric and myristic acids (present as 0.04% and 1.5–2.0% respectively) from oleic acid B.P.

KETO ACIDS

A quantitative separation and determination of the 2,4-dinitrophenyl hydrazones of keto-acids was worked out by Neish [3]. He employed Ford 428 Mill paper in an apparatus after Kunkel and Tiselius. Amongst numerous buffers tried, 0.05 *M* Na_2CO_3 was selected, and with 314 volt at 19° C for three hours the 2,4-dinitrophenyl hydrazones of the acids travel the following distances when placed on the paper near the cathode:

oxalacetic acid	14.6 cm
ketoglutaric acid	13.2 cm
pyruvic acid (i)	12.0 cm
pyruvic acid (ii)	9.6 cm

(i) and (ii) are two isomeric forms of the 2,4-dinitrophenyl hydrazones of pyruvic acid.

DI- AND TRI-CARBOXYLIC ACIDS

Wieland and Feld [4] employed *M*/50 acetate buffer at pH 7 for the separation of citric and tartaric acids. Both acids were detected by retention analysis with Cu^{++} ions.

Michl [5] also separated a pair of acids, malic and succinic, using a pyridine acetic acid buffer and detecting the acids with a mixture of KI, $KBrO_3$ and starch (gives a blue colour which fades rapidly). This work has been extended by Berbalk and Schier [6] and, using high voltages, by Gross [7].

References p. 74

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9. Carbohydrates and Hydroxycompounds

An early publication describes attempts to separate glucosamine and glucuronic acid from neutral sugars in a study of corneal mucopolysaccharides [1]. In this work 0.01 *N* NH_3, phosphate buffers at pH 4.3 and 9.2, and also 5 *N* acetic acid, were tried and yielded usually long trails of the amino sugar and of glucuronic acid.

The most important step in the development of electrophoretic methods for carbohydrates and hydroxycompounds was the use of borate buffers introduced by Consden and Stanier [2], Michl [3] and Jaenicke [4]. An extensive review of the chemistry of sugar-borate complexes was made by Zittle [5]. It was also pointed out in the paper by Michl [3] that borate complexes may be formed whenever an organic molecule possesses two adjacent OH-groups, and not only sugars but also polyhydric alcohols, polyphenols, their polymerisation products, and as shown by Hashimoto *et al.* [6], flavonoids may be separated.

As has been recently commented on by Gross [7], most workers emphasise the usefulness of high potentials for sugar separations. The various results will be classified into monosaccharides, disaccharides etc. but any over-lapping of compound class in the various publications will be retained.

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MONOSACCHARIDES

The most complete study of the separation of monosaccharides was made by Consden and Stanier [2]. An apparatus similar to that of Cremer and Tiselius (page 37) was employed with a borate buffer prepared by adding the required amount of 0.05 *M* borax to a solution of 0.2 *M* boric acid and 0.05 *M* NaCl. For pH higher than 8.6, mixtures of 0.05 *M* borax with 0.1 *M* NaOH were used.

The mobilities (cm^2/volt. sec.) were calculated from the distance between the sugar spot and a proline or creatinine spot and from the voltage, time and the effective length of the paper. The spots of the sugars were detected by drying and then spraying the paper with aniline hydrogen phthalate reagent, to which a few drops of glacial acetic acid had been added to overcome the alkalinity of the buffer. After a few minutes heating at 100–110°, the paper is viewed in ultra violet light in which the monose spots have an intense fluorescence. Alkaline picrate is used to reveal creatinine and ninhydrin for proline.

Table 6 shows the mobilities of sugars in borate buffers at various pH values. (See for Table 6, page 77).

Typical conditions used by Consden and Stanier are 10.3 volt/cm for 215 minutes at pH 8.6. A mixture of mannose, galactose and sorbose could be separated under these conditions into three distinct spots (Fig. 32). For complete separation and identification of all sugars present,

TABLE 6

MOBILITIES OF SUGARS (cm^2/V sec . 10^5) AT 20° IN BORATE AT VARIOUS pH VALUES ON WHATMAN No. 1 PAPER (CONSDEN AND STANIER [2])

pH	7.0	8.0	8.6	9.2	9.7
Fructose	8.2	9.7	11.4	12.5	13.1
Sorbose	8.7	10.4	12.2	14.1	14.3
Glucose	2.4	6.5	11.4	14.5	14.6
Galactose	2.8	5.8	9.6	13.0	13.1
Mannose	2.6	4.9	7.8	9.8	10.0
Ribose	7.0	9.1	10.2	10.9	11.0
Arabinose	3.2	6.5	10.3	13.3	13.9
Rhamnose	1.3	2.4	4.4	7.1	7.8
Cellobiose	0.5	0.5	1.5	3.2	4.5
Raffinose	0.5	0.9	1.7	3.6	4.8

paper chromatography in combination with electrophoresis is suggested. With these methods successful work on hydrolysates of human fibrin and group A haemolytic streptococcus hydrolysate is reported.

Hashimoto *et al.* [6] use borax solutions as electrolyte and record the distances travelled by various sugars using

TABLE 7

MOVEMENT OF SUGARS IN mm AFTER FOUR HOURS (HASHIMOTO, MORI AND KIMURA [6])

	I 500 volt-1% borax	II 650 volt–3% borax	III 700 volt–1% borax
Fructose	67	–18	–34
Glucose	56	–28	–47
Sorbose	46		–50
Galactose	84	–18	–52
Mannose	21	–15	–55
Rhamnose	3	–22	–45
Arabinose	21	–37	–51
Ribose	57		
Xylose	63	–19	–46

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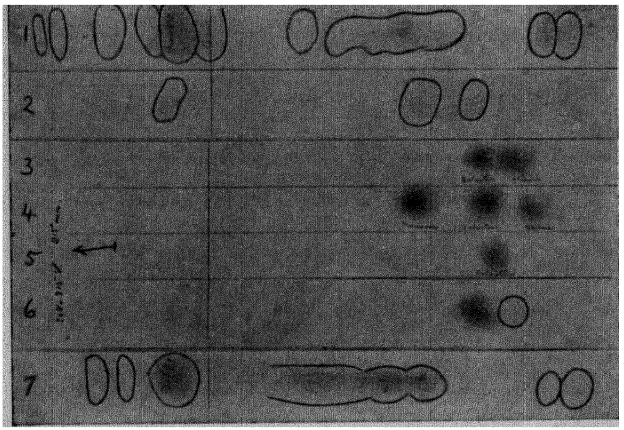

Fig. 32. Ionophoresis of protein hydrolysates and sugar mixtures in borate pH 8.6 at 10.3 volt/cm for 215 minutes. Origin on vertical line; anode at the right.

1 and 2 fibrin hydrolysate
3 galactose + glucose
4 mannose + galactose + sorbose
5 galactose
6 and 7 gelatin hydrolysate + galactose

Strips 1 and 7 were treated with ninhydrin; the two spots at the anode end are glutamic and aspartic acids. The other strips were treated with aniline hydrogen phthalate. The weaker spots are outlined. All the ionophoreses were carried out simultaneously on one sheet (Consden and Stanier [2]).

Durrum's method for four hours as shown in Table 7. See also Galos and Ostrowski [20] and Kariyone *et al* [22]. Briggs *et al.* [26] worked out a separation of glucose and mannose.

METHYL ETHERS AND DISACCHARIDES

An excellent discussion of the ionophoresis of methyl ethers of monosaccharides and disaccharides from the point of view of structural chemistry is to be found in the paper

by Foster[8]. He employs Whatman No. 3 paper with 0.2 *M* sodium borate buffer at pH 10 with a potential of 900 volt as standard conditions. The relative rate of movement of the sugars is expressed as the M_G value where

$$M_G = \frac{\text{true distance of migration of the substance}}{\text{true distance of migration of D-glucose}}$$

The true distances of migration are those corrected for movement due to electroendosmotic flow by reference to the movement of 2:3:4:6-tetramethyl glucose, which does not form a complex with borate ions. Table 8 gives the M_G values obtained for a large number of methyl ethers and disaccharides. See also Foster and Stacey[23].

TABLE 8

M_G VALUES OF METHYL ETHERS AND DISACCHARIDES (FOSTER[8])

Substance	M_G	Substance	M_G
D-Glucose	1.00	Melibiose	0.80
6-Methyl D-glucose	0.82	Gentiobiose	0.75
3-Methyl D-glucose	0.82	Isomaltose	0.69
3:5:6-Trimethyl D-glucose	0.71	Laminaribiose	0.69
3:4-Dimethyl L-rhamnose	0.36	Lactose	0.38
3:4-Dimethyl D-glucose	0.31	Maltose	0.32
4-Methyl D-galactose	0.27	Cellobiose	0.23
4-Methyl D-glucose	0.24		
2-Methyl D-glucose	0.23		
2:3-Dimethyl D-glucose	0.12		
α-Methyl-D-glucopyranoside	<0.09		
2:4-Dimethyl D-glucose	<0.05		
2:3-Dimethyl L-rhamnose	<0.05		
2:4-Dimethyl L-rhamnose	<0.05		
2:3:4-Trimethyl D-glucose	0.00		
2:3:6-Trimethyl D-glucose	0.00		

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Another list of M_G values of some glycopyranosides was given by Foster, Martlew and Stacey [9] as shown in

TABLE 9

M_G VALUES OF SOME GLYCOPYRANOSIDES (FOSTER, MARTLEW AND STACEY [9]).

Substance	M_G
α-Methyl-D-glucoside	0.11
β-Methyl-D-glucoside	0.19
1-Deoxy-D-glucose	0.20
α-Methyl-D-lyxoside	0.45
β-Methyl-D-lyxoside	0.26
α-Methyl-D-mannoside	0.42
β-Methyl-D-mannoside	0.31
α-Methyl-D-arabinoside	0.40
β-Methyl-D-arabinoside	0.40
1-Deoxy-D-arabinose	0.41

Table 9. For the separation of the dimethyl-L-rhamnopyranosides also see Foster [10]. More recently the separation of *n*-benzylglycosylammonium ions has been employed instead of borate complexes [25].

POLYSACCHARIDES

This is a group of compounds where chromatography has proved rather ineffective, while paper electrophoresis shows already great promise. An extensive study of the *acid mucopolysaccharides* has been published by Rienits [11]. An apparatus after Grassmann and Hannig was used with either Whatman No. 2 or Whatman No. 3 MM paper. As reagent, toluidine blue, or azure A solutions were used for qualitative detection. For quantitative estimations 1 cm strips were eluted by refluxing as in a Soxleth extraction

for about 2.5 hours and the reaction of Morgan and Elson [12] or the carbazole method of Dische [13] were subsequently employed. Synthetic mixtures of hyaluronic acid, chondroitine sulphate and heparin were separated with 0.1 *M* sodium phosphate buffer at pH 6.7 in four hours with a potential gradient of 5 volt/cm. Hyaluronic acids

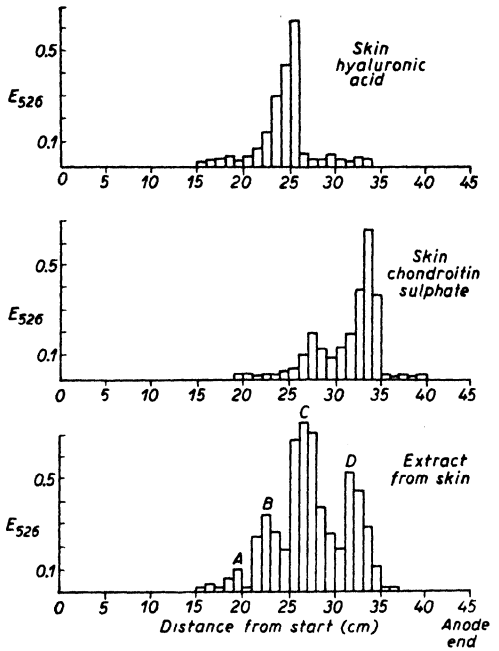

Fig. 33. The electrophoresis in 0.1 *M* sodium acetate buffer at pH 5.0 of various skin preparations, each representing material obtained from approximately 10 g skin. The acid mucopolysaccharides were detected by elution of the strips followed by hexosamine determination. (Ordinate, optical densities at 526 mμ) (Rienits [11])

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from various sources before and after NaOH treatment were examined; they move at different speeds (0.1 *M* acetate buffer at pH 5.0 for 17 hours with 3.8 volt/cm). Finally it was demonstrated that an extract from skin can be separated into four peaks as shown in Fig. 33, using 0.1 *M* acetate of pH 5.0 as before.

Galactogen and glycogen were separated on silk instead of paper; for details see Geldmacher-Mallinckrodt and May [14]. For iodine complexes of hydrolysis products of amylose see Mould and Synge [21].

Koiw and Grönwall [15] detect the mucoproteins and glycoproteins in serum by bathing the paper strips in periodic acid, washing with 70 % alcohol, reducing with KI-$Na_2S_2O_3$ solution and finally dyeing with fuchsin in acid bisulphite solution. This treatment yields violet red bands for all polysaccharides while the paper produces only a weakly dyed background. Direct photometry can be carried out with this technique. For clinical applications see the paper of Wunderly and Piller [16].

POLYHYDRIC ALCOHOLS AND PHENOLS

Michl [3] examined the behaviour of glycol, glycerine and mannitol in 0.08 *M* borax solution. As reagent, lead tetraacetate in benzene was used. A good separation of mannitol (mobility 6.0) from glycerol (mobility 3.5) was obtained; glycol could not be detected.

Foster and Stacey [17] and Foster [19] report the M_G values

TABLE 10 [17]

Substance	M_G value	*Cis* OH groups
(—)-Inositol	0.69	C_1:C_2 , C_5:C_6
(+)-Inositol	0.69	C_1:C_2 , C_5:C_6
Pinitol	0.66	C_1:C_2 , C_5:C_6
meso-Inositol	0.53 (diffuses extensively)	C_1:C_2:C_3
Quebrachitol	0.31	C_5:C_6
(+)-Bornesitol	0.12	C_2:C_3

TABLE 11 [19]

Substance	M_G value	*Cis* OH groups
meso-Inositol	0.51	C_1:C_2:C_3
1:2:3-Cyclohexanetriol	0.19	C_1:C_2:C_3
1:2/3-Cyclohexanetriol	0.10	C_1:C_2
Sequoyitol A	0.18	C_1:C_2:C_3
Sequoyitol R	0.00	
(+)-Inositol	0.63	C_1:C_2 , C_5:C_6
(—)-Inositol	0.63	C_1:C_2 , C_5:C_6
epi-Inositol	0.73	C_1:C_2:C_3:C_4:C_5
allo-Inositol	0.88	C_1:C_2:C_3:C_4 , C_5:C_6
muco-Inositol	0.96	C_1:C_2 , C_4:C_5
scyllo-Inositol	0.05	

(see page 79) of a number of cyclitols and their methyl ethers. In Tables 10 and 11 the M_G values are obtained under the following conditions: 0.2 *M* borate at pH 10 using Whatman No. 3 paper with 900 volt for 1.5 hours. The apparatus is essentially that of Kunkel and Tiselius with additional water cooling on the lower glass plate (Foster [18]).

Some of the cyclitols appear in both tables with slightly different M_G values, as these are still more relative than absolute and in each table the difference and sequence is

more reliable than the rate of migration with reference to D-glucose. For further work see Gross [24].

Amongst the phenols those having adjacent OH groups (*o*-diphenols), such as gallic acid methyl ester and pyrogallol, all travelled at about the same speed (mobility 5.7–5.9) and thus could be separated from monophenols and other polyphenols but not from each other. The electrolyte used for the phenols was 0.11 *M* borax–0.04 *M* sodium sulphite. Electrophoresis on paper is also suggested for the analysis of commercial tannins (Michl [3]).

FLAVONOIDS

A number of flavonoids have been examined by Hashimoto *et al.* [6]. Using Durrum's method, 2 % borax and 500 volt for four hours, they report the distances moved as shown in Table 12.

TABLE 12

THE MOVEMENT OF SEVERAL FLAVONOIDS WITH 500 VOLT, 2% BORAX FOR FOUR HOURS EXPRESSED IN mm (HASHIMOTO *et al.* [6]).

Flavonoid	Number of *cis* OH of sugar	Number of vicinal OH in aglycone	Distance moved towards cathode in mm
Myricitrin	1	1	30
Rutin	1	1	25
Quercetin	0	1	16
Myricetin	0	1	10
Lutedin-7-glucoside	0	1	3
Maringin	0	1	3
Hesperidin	0	0	3
Acacetin	0	0	3
Morin	0	0	2
Robinin	0	0	2

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10. Nitrogen Compounds

AMINES

An extensive study of the separation of amines was made by Weber [1] employing a special apparatus of the horizontal type. With a paper strip 150 cm long, and with 1300 volt and a citrate buffer at pH 3.8 (10.5 g citric acid per litre adjusted to pH 3.8 with NaOH), he obtained a complete separation of the following: (slowest) glycine, mescaline, phenylethylamine, histamine, cadaverine, dimethylamine, monomethylamine (fastest).

Weber further measured the relative rates of travel of a large number of amines and expressed their movement with relation to the movement of NH_4^+ and K^+.

Table 13 (page 87) shows the order obtained.

AMINOSUGARS

Blass *et al.* [2] studied the separation of glucosamine from some amino acids and amines. The following order is given with paper strips 14 × 44 cm, 10 v/cm with pH 8.6 veronal sodium buffer for $3\frac{1}{2}$ hours:

— lysine +
ethanolamine – arginine – glucosamine – serine – cystine – aspartic acid.

INDOLE DERIVATIVES

The natural indole derivatives from plant extracts were

TABLE 13

RELATIVE RATE OF TRAVEL OF AMINES (COMPARED WITH THE SPEED OF K^+ AND $NH_4^+ = 1$)

Substance	Rate of travel
Urea	0
Glycine	0.04
Mescaline	0.24
Arginine	0.25
Histidine	0.26
Lysine	0.26
Gramine	0.27
Ornithine	0.28
Tyramine	0.29
Ephedrine	0.32
Creatine	0.37
Phenylethylamine	0.41
Histamine	0.58
Propylamine	0.61
Cadaverine	0.64
Putrescine	0.67
Ethylamine	0.72
Dimethylamine	0.81
Methylamine	0.92
NH_4^+, K^+	1.0

examined by Denffer *et al.* [3, 4] using the Elphor apparatus with pH 7.0 phosphate buffer for 8 hours at 110 volt. Four bands were separated from a corn extract and β-indoaceto-nitrile detected in an extract from cabbage. See also A. Fischer [24].

SULPHANILAMIDES

The movement of sulphanilamides when mixed with human serum and in pure solutions was studied by Kutzim [5] who found that the presence of serum in the initial

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bands does not change the movement of the sulphanilamides in any way. Using the Elphor apparatus with pH 8.6 Michaelis buffer and 110 volt, he records the relative rates of movement as shown in Table 14. Okac and Jokl [28] studied various buffers ranging from pH 1–13.

TABLE 14

RELATIVE RATES OF MOVEMENT OF SULPHANILAMIDES (KUTZIM [5])

Badional	1.0
Albucid	1.0
Gantrisin	0.83
Cibazol	0.82
Globucid	0.80–0.81
Methylpyriminal	0.74
Elkosin	0.64
Eubasin	0.37
Prontosil	0.39
Prontalbin	0.02
Urea	0.00
p-Aminobenzoic acid	1.13

For phenobarbitones and aminopyrine and derivatives see Kinoshita and Moriyama [29, 30] and Wagner [31].

AMINO ACIDS AND PEPTIDES

Amino acids have been extensively studied by various workers, but unfortunately only a few papers concern themselves with separations of more than a few amino acids. As the analysis of amino acids from natural products would require isolation of up to twenty-five amino acids, several new techniques were also employed and will be discussed here.

Wieland and Fischer [6] separated glutamic acid, alanine

and histidine at pH 5; at pH 7.5 histidine is isoelectric but lysine moves towards the cathode; at pH 3.7 glutamic acid is isoelectric and aspartic acid travels to the anode.

Copper complexes of amino acids were separated by Wieland *et al.*[7]. By employing radioactive copper a quantitative estimation by the measurement of the radioactivity of the spots was evolved.

Biserte[8] effected the separation of natural mixtures of amino acids in a special apparatus with 360 volt in *M*/15 phosphate buffer at pH 7.2 into five fractions in three hours:

on the cathode side	1 arginine
	2 glycine, threonine, alanine, histidine and proline
	3 traces of aspartic and glutamic acids
on the anode side	4 and 5 aspartic and glutamic acids

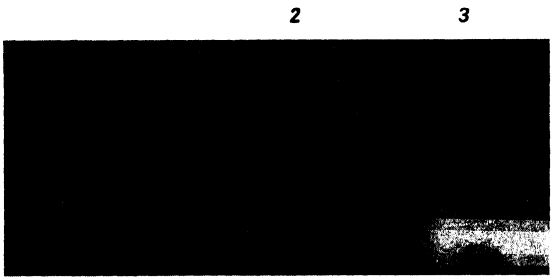

Fig. 34. Separation of amino acids with pH 8.2 veronal buffer, 300 volt in 2hr, using a Durrum apparatus and No. 1 Whatman paper (from the laboratory of Dr. E. Lederer). The amino acid mixtures are obtained from the hydrolysis of bacterial phosphatides.
1 glutamic acid
2 neutral amino acids
3 basic amino acids

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For separations of amino acids and peptides, see also Biserte [8]; for a study of the separation of basic amino acids, Harris and Warren [9].

Figures 34 and 35 show some separations obtained with pH 8.6 veronal buffer. Grassmann and Hannig [10], also Grassmann [11] effected numerous separations by continuous electrophoresis, thus the following pairs and groups were separated at the pH values stated:

aspartic acid – glutamic acid (citrate buffer at pH 3.79)
glutamic acid – alanine – histidine (M/15 phosphate at pH 5.2)
lysine – histidine (pH 6.8)
leucine – glutamic acid (pH 5.2)
glycine – alanine (pH 11.5)
alanine – threonine (pH 11)
alanine – cystine (pH 11.5)
arginine – histidine – alanine – aspartic acid (pH 7.4)
lysine – alanine – threonine – glutamic acid (pH 8.5)

In the publications of Durrum [12–14] the most complete data on amino acids are presented: A mixture of 19 amino

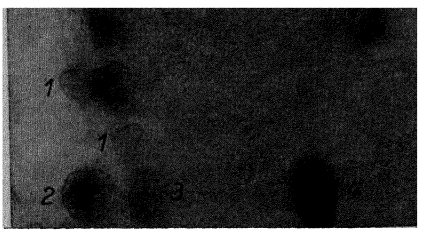

Fig. 35. Separation of amino acids. Conditions as in Fig. 34 (from the laboratory of Dr. E. Lederer).
1 hydrolysates of phosphatides
2 lysine
3 hydroxylysine
4 alanine

acids separated with potassium phthalate buffer at pH 5.9 into five bands in two hours with 600 volt: (i) arginine and lysine, (ii) histidine, (iii) monoamino-monocarboxylic amino acids, (iv) glutamic acid and (v) aspartic acid. At pH 1.7 (in 5 *N* acetic acid) with 580 volt in two hours a complete separation of a mixture of alanine, valine, proline and tryptophan, also of a mixture of glycine, isoleucine, phenylalanine and hydroxyproline was achieved. A mixture of glycylglycine and glycyl-L-leucine also separated in 5 *N* acetic acid.

TWO-DIMENSIONAL SEPARATION OF AMINO ACIDS

Electrophoresis on square sheets of paper, first along one axis with a volatile buffer, then along the other axis with a different buffer, has been used by Durrum [14] for amino acid separation. The most effective electrolytes are 0.2 *N* NH_4OH (pH 11.3) and 1 *N* acetic acid (pH 2.3). On S & S No. 413 paper, the order of movement in these electrolytes is as shown in Table 15.

Members of groups singly spaced in these lists do not separate from each other. It is evident from the various lists of sequences at different pH values that good separations of all amino acids are feasible by either two-dimensional or continuous methods followed by another separation. One possibility not yet explored would be to use several continuous apparatuses in series with different pHs in the background electrolyte. Such an arrangement

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TABLE 15

ORDER OF MOVEMENT OF AMINO ACIDS IN TWO-DIMENSIONAL SEPARATION

1 *N* acetic acid (pH 2.3)	0.2 *N* NH_4OH (pH 11.3)
To cathode	To anode
lysine	aspartic acid
histidine	glutamic acid
arginine	cystine
glycine	glycine
	serine
alanine	threonine
	hydroxyproline
valine	tyrosine
isoleucine	methionine
leucine	histidine
	phenylalanine
serine	alanine
threonine	valine
methionine	isoleucine
tryptophan	leucine
	tryptophan
phenylalanine	
glutamic acid	proline
tyrosine	lysine
proline	arginine
cystine	
aspartic acid	
hydroxyproline	

carried out on an industrial scale should prove worth while for the commercial preparation of pure amino acids from waste products such as from wool or silk.

McDonald *et al.* [15, 16] use amino acids in various studies of theoretical interest for their apparatus (see page 33) and also separated several pairs of amino acids at various pHs.

Electrophoresis with a *pH gradient* at right angles to the electric field was described by Michl [17] who used the pH range from 4.0–5.0 and demonstrated the different degrees of separation obtained when a mixture of arginine, lysine, alanine, glycine, glutamic acid and aspartic acid is electrophorised. The pH gradient is produced by impregnating the paper by ascending flow with pyridine acetic acid

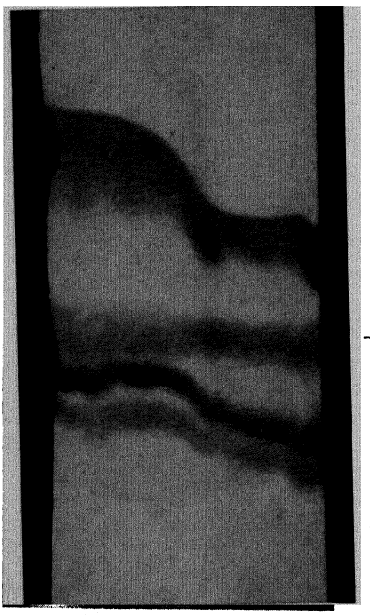

Fig. 36*a*. Separation of arginine, lysine, alanine, glycine, glutamic acid and aspartic acid with a pH gradient. The pH gradient is shown in the schematic representation.

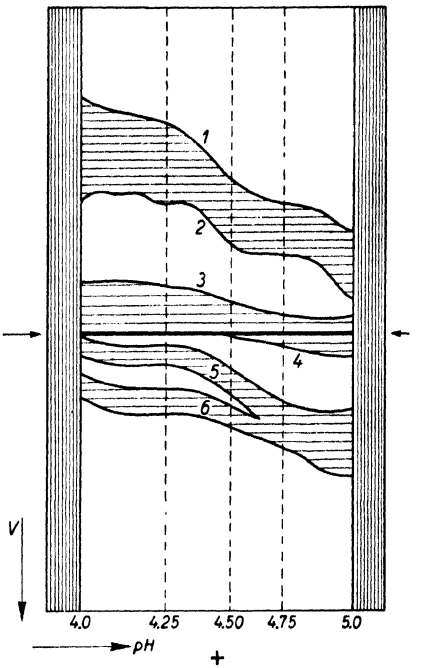

Fig. 36*b* (after Michl [17]).
1 arginine 4 glycine
2 lysine 5 glutamic acid
3 alanine 6 aspartic acid
The arrows indicate the starting position.

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buffer, first from one side and then from the other side with a different pH buffer. Diffusion produces a reasonably uniform gradient as shown in Fig. 36.

DNP-AMINO ACIDS

DNP-amino acids were separated by Biserte and Osteux[18] using a *M*/30 phosphate buffer at pH 7.0 with 400 volt for 22–24 hours (special apparatus with long paper). Four spots were separated: (i) DNP-aspartic and glutamic acids, (ii) DNP-monoamino-monocarboxylic acids, (iii) ε-di-DNP-lysine and di-DNP-histidine and (iv) ε-DNP-lysine and α-DNP-arginine. Further separations were also achieved after employing special reactions in some cases.

GUANIDO DERIVATIVES

Lissitzky, Garcia and Roche[26] separated the guanido derivatives and related compounds with veronal buffer pH 8.6 with 300 volt on Arches 302 paper in two hours. They record the following distances migrated:

citrulline	3.20 cm
glycocyamine	3.85 cm
creatine	4.00 cm
γ-guanido-butyric acid	4.00 cm
arginine	8.05 cm
arcaine	10.70 cm
dimethylguanidine	12.85 cm
agmatine	13.00 cm
methylguanidine	13.9 cm
guanidine	14.9 cm

PEPTIDES

For separation of biologically important peptides, electrophoresis on paper was successfully used by Taylor *et*

al. [19] in the case of commercial vasopressin and oxytocin preparations. Beef vasopressin separated into four spots in acetate buffer at pH 5.7.

Conflicting evidence has been presented on the properties of ACTH preparations. McDonald *et al.* [20] separate an extract into three bands with veronal buffer at pH 5.5 with 6 volt/cm in three hours. They report that 98 % of the activity is associated with a spot, with an isoelectric point between pH 6–6.6.

Li [21], working with an extract obtained from sheep pituitaries, using the Tiselius and Kunkel method and correcting for electro-osmotic flow reports the isoelectric point of the active fraction to be pH 9. The divergence of results appears rather due to differences in technique and methods of determining the isoelectric point. See also Loomeijer and Witter [22] and Macek *et al.* [25]. For remarks on the paper electrophoresis of a polyglutamyl peptide see Strange and Harkness [23].

IODOTHYRONINES AND IODOTYROSINES

Lissitzky [27] separated the iodothyronines with NaOH 0.012*N* (in 2% glycerol) in 7 hours and 300 volts. 3,5-Diiodothyronine moves 4.0 cm, 3,5,3'-triiodothyronine 7.7 cm and thyroxine 10.7 cm. The iodotyrosines were separated with pH 8.6 veronal buffer, which separated tyrosine, iodotyrosine and diiodotyrosine, while the iodothyronines remain on the point of application.

For iodo-proteins see page 125.

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11. Alkaloids

Pairs of alkaloids were separated by Deckers and Schreiber [1] using, for the separation of scopolamine and hyoscyamine *N*/5 borate buffer at pH 8.6 for two hours, and for brucine and strychnine a citrate buffer at pH 3.5. These workers also used the continuous technique with S & S 2040a paper, and were able to separate 100 mg of a scopolamine-hyoscyamine mixture in 40 hours with 160 volt and a pH 8.6 veronal buffer.

Burma [2] examined numerous alkaloids in buffers at vari-

TABLE 16

MOVEMENT OF ALKALOIDS AND OTHER BASES IN 5 *N* ACETIC ACID WITH 750 VOLT, 0.5 mA/cm IN 2 HOURS (KARIYONE *et al.*[3])

Substance	Migration distance	Substance	Migration distance
Aconitine	32	Hydrastine	73
Acrinol	39	Hyoscyamine	46
Aneurine	72	Lobeline	46
Atebrin	50	Neoheteramine	46
Atropine	45	Nicotine	99
Banthine	64	Oxine	74
Benadryl	46	Pelletierine	80
Berberine	41	Plasmochine	65
Brucine	40	Procaine	47
Caffeine	5	Quinine	74
Chelerythrine	37	Sanguinarine	34
Cinchonine	80	Sinomenine	43
Coniine	105	Solanine	34
Emetine	46	Strychnine	48
l-Ephedrine	52	Sparteine	76

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ous pH values without achieving noteworthy results. Thus, he found that alkaloids within a certain group do not separate well and suggests paper electrophoresis mainly for the separation of various groups of alkaloids.

Kariyone *et al.* [3] also carried out an extensive investigation with common alkaloids, and obtained good separations under various conditions. Table 16 shows the

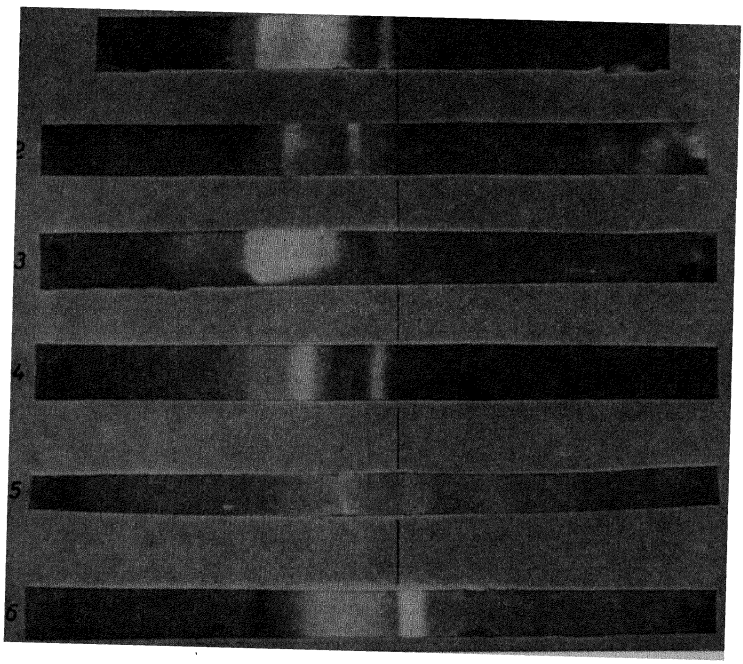

Fig. 37. Separation of curare alkaloids. The six bands show the ultraviolet fluorescent substances from six different *Strychnos* varieties. 300 volt, 1 hr, with 25 % acetic acid as electrolyte (Marini-Bettolo, private communication).

distances moved by a number of alkaloids when electrophorised with 5 *N* acetic acid as electrolyte.

Marini and Lederer[4] used paper electrophoresis for the study of curare alkaloids such as those derived from *Strychnos trinerva*. Ceric sulphate spray and ultra-violet fluorescence were used to detect some of the bands. Figs. 37 and

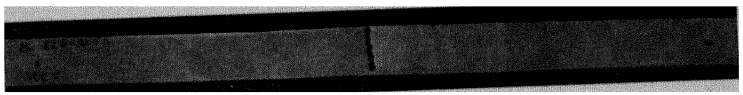

Fig. 38. Separation of the alkaloids of *Strychnos trinerva* with 2 % borax as electrolyte with 300 volt for 1 hour (viewed under ultra-violet light; Marini-Bettolo and Lederer [4]).

38 show the separation of bands, fluorescent under ultra-violet light, as obtained with 5 *N* acetic acid and with 2 % borax for one hours with 300 volt.

Scopolamine, dihydroxycodeine and ephedrine were separated in that order by Macek and Trčka [5] using veronal pH 9.4 buffer with 100 volt for 4 hours. The spots were detected with Dragendorff"s reagent. For the separation of morphine and chelidonine see Graf and List [6].

Urine of drug addicts was examined by Sano and Kajita [7].

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12. Proteins

The first to effect a qualitative separation of serum proteins were Durrum [1] and Turba and Enenkel [2] Tiselius and Cremer [3] published the first quantitative estimation a little later. Since then, numerous articles have appeared on methods of separation, estimation and clinical applications. In this chapter the compilation of many clinical researches in a review by Wunderly [4] will be referred to extensively. The literature dealing with protein separations is found in journals on biology and chemistry as well as in various medical journals and will not be reviewed completely.

DETAILS OF THE ELECTROPHORETIC TECHNIQUES

In Table 17 are the variables of the electrophoretic separations used by some authors. Usually 0.015–0.04 ml of serum are placed on the paper, either as a line or a round spot. Macheboeuf [5] recommends dilution of the serum with isotonic saline. Graf and List [6] consider dialysing against the buffer important. However in most clinical work the serum is placed directly on the paper.

Buffers

The buffers most commonly employed are those previously used in free electrophoresis such as 0.05 *M* veronal

TABLE 17

VARIABLES OF ELECTROPHORETIC SEPARATIONS OF PROTEINS

Author	Filter paper	Dimensions in cm	Potential in volts	Current in mA	Time hours	Distance of separation in cm
Durrum [1]	Whatman No. 2	1 × 32	220–320	0.5	3	ca. 3.5
Macheboeuf *et al.* [5]	Whatman Nos. 1 and 2	6 × 32	320–340	3	6	16–18
Flynn and de Mayo [8]	Whatman Nos 1 and 3 MM	6 × 36	120	1.5	20	16–18
Wunderly *et al.* [9]	Munktell 20/150	7 × 40	135	6–7	9	8–9
Grassmann and Hannig [10]	Whatman No. 1	4 × 40	110	4	14	9–10
Cremer and Tiselius [3]	Munktell 20	4.3×35	140	1.8	12	23
Schneider [11]	Munktell 20	4 × 40	190	2.6	12–16	20

buffer at pH 8.6 [3], or veronal/acetate buffer pH 8.6 of ionic strength 0.1 prepared by diluting 29.34 g of veronal sodium, 19.42 g sodium acetate trihydrate and 180 ml of *N*/10 HCl to 3 litres with distilled water (Wunderly [4]), or a similar barbiturate buffer (Durrum [1]). These buffers are being used in most analytical work although Consden [137] and also Wieland and Wirth [23] found that the much cheaper borate buffer can be used equally well for serum proteins. Holt *et al.* [7] use a lower ironic strength 0.045 and a high potential for separations in two hours. For

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pH 6.4 a 0.033 *M*. Soerensen phosphate buffet and for pH 3.7 a 0.1 *M* sodium acetate/acetic acid buffet have been used. See also Berkes and Karas [91] on the influence of the conductivity of the buffet on the degree of separation.

Dyeing

After the moist paper strips are taken from the electrophoresis chamber, they are usually dried in an air oven at 100° C. Recently it was shown that drying is not important when bromophenol blue is subsequently used.

(1) *Bromophenol blue.* Durrum [1] dipped the dried paper in a 0.1 % bromophenol blue solution in ethanol saturated with mercuric chloride. The excess dye is removed by washing exhaustively in running water. Cremer and Tiselius [3] modified this procedure to render the amount of dye held on the paper reproducible for quantitative estimation by dipping into the above bromophenol blue solution and then washing the paper successively with the following: 1 % $HgCl_2$ in methanol, 1 % $HgCl_2$ in ethanol, methanol and ether.

Kunkel and Tiselius [12] wash the paper with four changes of 0.5 % acetic acid for 20 minutes each, as in an acid medium bromophenol blue is more strongly adsorbed on the protein. The blue colour is then estimated as discussed below.

(2) *Azocarmin B.* Turba and Enenkel [2] dye for 10 min-

utes with a mixture of 50 ml methanol, 10 ml acetic acid (85 %) and 40 ml of a saturated aqueous solution of azocarmin B. The paper is then washed for 5 minutes in methanol and for 5 minutes in 10 % acetic acid. Wunderly [4] claims that this dye stains all fractions with about the same intensity.

(3) Naphthalene black 12*B* 200 (*in German*: *Amidoschwarz*). Grassmann and Hannig [10, 13] dye for 10 minutes with a saturated solution of this dye in methanol containing 10 % acetic acid. The strips are then washed several times with methanol containing 10 % acetic acid.

(4) *Benzidine*. Reindel and Hoppe [14] report a method suitable for dyeing amino acids, peptides and proteins. The paper is dried at 80°, dipped into an ethanol-acetone (1:1) mixture containing 0.5 % acetic acid, and then exposed to an atmosphere of ClO_2. The paper is then placed in a solution of benzidine in 10 % acetic acid till a maximum development of colour is obtained and then washed three times with alcohol.

QUANTITATIVE EVALUATION OF PROTEINS ON THE PAPER

The numerous techniques of quantitative estimation mentioned below were developed to produce an "electrophoretic pattern" similar to that of the Tiselius free electrophoresis. The normal amount of the globulins is relatively small and the accuracy of their determination, both in Tiselius and paper methods, not great. Alterations in

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pathological conditions are usually quite considerable and can be detected by visual examination of the pattern or the paper strips. Thus in the author's opinion, the value of precise quantitative methods for clinical purposes is still questionable in most cases. Latner and Richardson [16] describe a visual matching technique for paper strips. Paper electrophoresis followed by various quantitative techniques has however already become an established laboratory method in clinical chemistry.

Elution and colorimetry of the dye

In the first method for the quantitative evaluation of protein stained with bromophenol blue [3], the paper is cut into 5 mm wide strips at right angles to the separation direction and each strip placed in a test tube with 3 ml (or at high intensities 6 ml) of a Na_2CO_3 solution (50 ml methanol, 50 ml 10 % aqueous Na_2CO_3 solution). After 30 minutes the extraction of the dye is complete, and the blue colour is measured with a Beckman spectrophotometer at 595 mμ. Various proteins differ in their absorption of dye, albumin absorbing more than the globulins. Cremer and Tiselius [3] multiply the globulin absorption by a correction factor of 1.6. More precise measurements of these corrections for bromophenol blue were made by Kunkel and Tiselius [12], for naphthalene black 12B 200 by Grassmann and Hannig [10], and for both dyes by Sommerfelt [17]. See also Koiw, Wallenius and Grönwall [18]. Esser *et al.* [19] correct differently for each globulin, taking albumin as unity,

they multiply $alpha_1$ by 2.36, $alpha_2$ by 2.09, beta by 2.20 and gamma by 1.62. See also De Jong [127].

Direct photometry of the paper strip

Numerous commercial instruments for direct photometry of paper chromatograms and electropherograms are available.

We shall only mention the recording photo-densitometer of Joyce, Loebl and Co. Ltd. (Newcastle-upon-Tyne, England), the Elphor photometer (Bender and Hobein, Munich, Germany), the photometer of Jouan (Paris), the recording photometer of Leres (Paris) and the automatic photometer of Kutacek and Kolousek (Czechoslovakia) [21]. See also Cooper and Mandel [113].

Grassmann *et al.*[10, 20, 109] at first considered it necessary to dip the paper into a liquid with a refractive index near that of paper (1 part bromonaphthalene and 1 part paraffin oil) for an accurate evaluation. Later workers dispensed with this and have obtained quite satisfactory quantitative results. Often instruments for direct photometry are mounted on or sold together with the electrophoresis apparatus.

Eisenreich and Eder [22] embed the strips in xylene, after bleaching with oxalic acid, for subsequent photometry. For clinical results, photometry appears to be more suitable than the tedious elution techniques. Critical comparisons of quantitative methods were made by Fuchs and Flach [125] and Van Kampen and Zondag [126]. See also Wunderly [129].

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Retention analysis

After staining with azocarmin B and drying, Wieland and Wirth [23] allow a solution of copper acetate (100 mg) in tetrahydrofuran (100 ml containing 7 % water and 5–10 drops acetic acid) to ascend the paper at right angles to the separation direction. A notch in the Cu^{++} front, proportional to the Cu^{++} adsorption of the protein, is formed at each band and this is revealed by drying and spraying with 0.5 % rubeanic acid in acetone.

Fig. 39. Retentiometric detection of the fractions of cobra venom (Wieland and Wirth [23]).

The pattern obtained with cobra venom is shown in Fig. 39. The quantities are calculated by the expression

$$\frac{\alpha + \beta}{\alpha} = \frac{h}{y}$$

where α = the retention of Cu^{++} by the paper
β = the retention of Cu^{++} by the protein
h = the height of Cu^{++} on the paper (without protein)
y = the height of Cu^{++} on the paper holding the protein band.

The main disadvantage of this method is evident from Fig. 39, namely that the edge of the Cu^{++} front is so serrated as to make the detection of minor bands difficult.

A method which produces a pattern similar to that of

free electrophoresis has been worked out by Michl [15]. He places the dyed paper strip or its photographic negative in front of an optical system of two cylindrical lenses and a grey wedge as shown in Fig. 40. This produces a pattern of maxima for the dyed bands, whose surface area is pro-

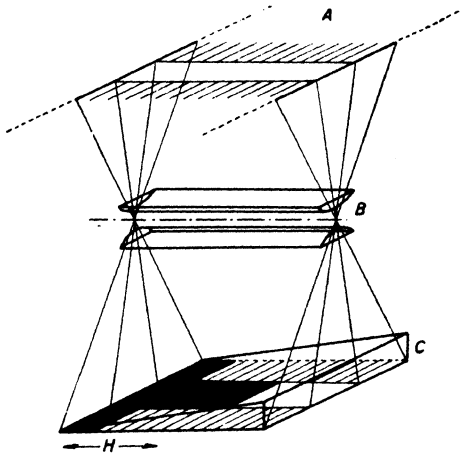

Fig. 40. Optical system of Michl [15] to yield patterns as in free electrophoresis.

A negative of the paper strip
B cylindrical lenses
C grey wedge

portional to the quantity of the dye. Thus for direct quantitative estimation of the areas, a dyestuff which dyes all bands equally is necessary. Michl suggests a mixture of Neucoccin and Naphtholblauschwarz B and copper or nickel acetate. The colour is deepened by washing with

rubeanic acid. The pattern obtained for the egg white proteins is shown in Fig. 41.

Ultra-violet colorimetry

Kimbel [24] found that in ultra-violet light around 250 mμ it is possible to measure the absorption of the various

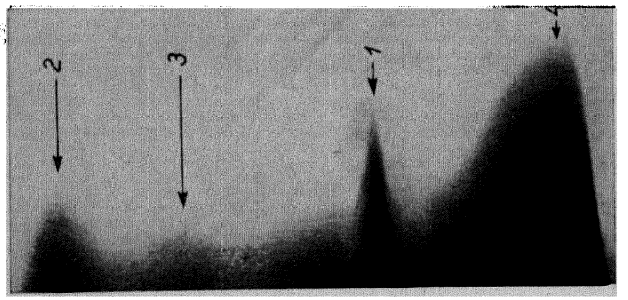

Fig. 41. Pattern of egg white proteins prepared with the apparatus of Michl [15].
1 point of application
2 conalbumin
3 globulins and ovomucoid
4 ovalbumin

proteins. An instrument was designed which records the absorption curves automatically. Comparisons with other methods have not as yet been published.

The above methods exhaust neither the possibilities nor the number of methods proposed. For example Levin and Oberholzer [25] propose a Kjeldahl determination on each of the separated bands; see also Anderson [26]. The biuret reaction is proposed by McKinley *et al.* [92]. The techniques

here mentioned should be sufficient information for the development of a method suitable to a specific problem.

Accuracy of the quantitative methods

Numerous articles [10, 11, 17, 21-36] concern themselves with the comparison of the Tiselius and the paper electrophoretic methods. In both, the accuracy of the estimation of the globulin fractions is of the order of ± 10% and the albumin of the order of ± 3%.

TABLE 18

COMPARISON OF PAPER AND OPTICAL ELECTROPHORETIC METHODS WITH NORMAL SERA BY SCHNEIDER [11] EXPRESSED IN % OF THE TOTAL PROTEIN

		Paper electrophoresis				Optical electrophoresis			
	No.	Alb.	α	β	γ	Alb.	α	β	γ
Series 1	1	63.1	10.1	9.8	17.0				
	2	65.9	10.6	10.3	13.2				
	4	64.4	7.7	9.3	18.6				
	8	66.3	10.1	11.2	12.4				
Mean		64.9	9.6	10.2	15.3	61.5	8.1	12.1	18.3
Average error of the mean		±0.7	±0.7	±0.4	±1.5				
Series 2	14	46	16	22	16				
	15	55	9	16	20				
	17	60	12	10	14				
	28	55	11	14–6	14				
Mean		54	12	17	17	60.8	9.5	15.0	14.7
Average error of the mean		±3	±1.4	±2.8	±1.3				

Typical results of a comparison of the optical (Tiselius) and the paper strip method, with evaluation of the bromophenol adsorption, are shown in Table 18, from Schnei-

TABLE 19

COMPARISON OF RESULTS WITH NORMAL SERA BY VARIOUS TECHNIQUES AND AUTHORS (AFTER WUNDERLY [4]).

Author	Plasma P Serum S	No. of cases	Age	Method	Alb.	α_1	α_2	β	γ	φ Fibrinogen
eibert *et al.* [27]	S	43	18–55	optical method Tiselius-Philpott-Svensson	53.3	8.0	10.4	13.8	14.2	
afsky, Brill, Stern	P	13	3–8		57.7	6.3	10.6	10.3	9.7	5.2
and Corey [28]	S & P	30	9–16		58.8	4.9	9.5	12.6	9.5	5.3
	P	15	19–25		60.3	4.6	7.2	12.1	11.0	5.1
	S	80	18–55		56.0	7.2	8.8	13.1	14.7	
	S	31	65–95		48.6	8.5	10.2	16.7	15.9	
Hartmann and Schumacher [29]	S	130		Antweiler	63.3	6.0	5.8	13.1	11.5	
Verbeck [30]	S	20		Antweiler	63.2	9.0		13.7	14.1	
Grassmann and Hannig [10]	S	25		Paper	61.3	4.1	8.1	11.0	15.5	
Glückthun and Götting [31]	S	15		Paper	59.0	4.2	8.0	10.6	18.2	
Löiw, Wallenius and Grönwall [32]	S	10		Paper	72.9	1.4	3.5	8.6	13.6	
Gras [33]	S	26		Paper	66.6	10.1		8.7	14.6	
Caspani and Bernasconi [34]	S	11		Paper	62.7	3.9	7.5	10.7	14.9	
Goa [35]	S	17		Paper	59.2	3.8	6.0	11.1	19.6	
Brante [36]	S	39		Paper	64.9	7.6		11.1	16.4	

der [11]. Table 19 compiled by Wunderly [4] lists normal values for sera measured by various techniques. Both tables show that paper electrophoresis produces higher albumin and lower globulin values, especially the lipoid-carrying alpha and beta globulins. See also Pieper and Molinski [108]. Tizzani [131] carried out a statistical study of normal sera.

DETECTION OF LIPOIDS IN SERUM FRACTIONS ON THE PAPER

The detection of lipoids in the various fractions on the paper after electrophoresis has been found of interest both in clinical diagnosis and investigations of protein-lipid relationships.

Swahn [37] dyed the dried strips in a half-saturated solution of Sudan black in 50 % ethanol for 30–45 minutes. Durrum *et al.* [38] found the dye oil red O (formula below) superior to any other dye examined by them. They stained the strips by immersing for 16 hours in a bath of a saturated solution of oil red O in 60 % ethanol, rinsing under the tap and blotting and drying (Fig. 42). Wunderly and Pezold[39] employ a mixture of dyes as follows: 20 mg of Sudan black and 20 mg of Ciba Blau are dissolved in 40 ml of warm ethanol and, while stirring, 40 ml of 2 % Na_2CO_3 gradually added. The solution is filtered warm and used immediately for the dyeing of 6–8 strips. The strips are left in the solution for 6 minutes, then washed with cold 50 % ethanol three times, allowing twenty minutes after the first and thirty minutes after the second wash. After

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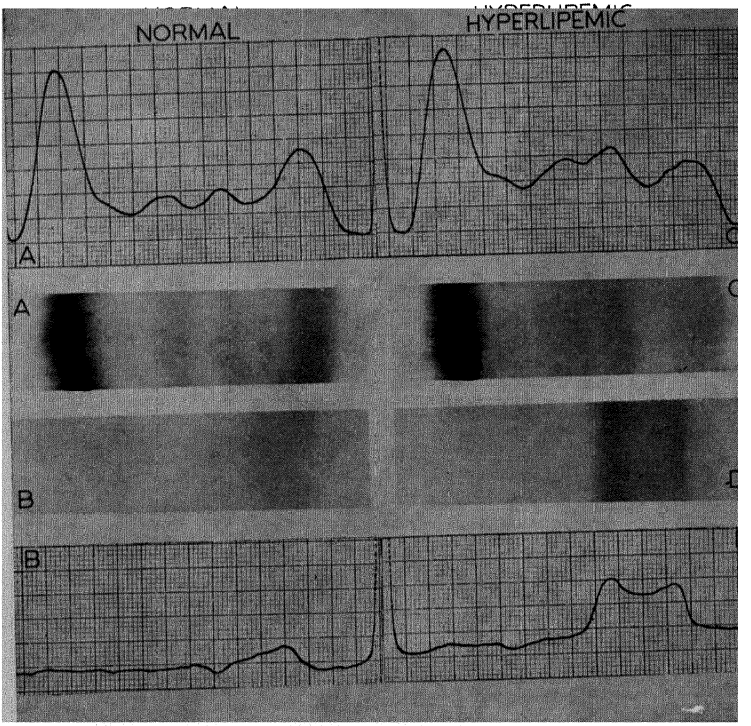
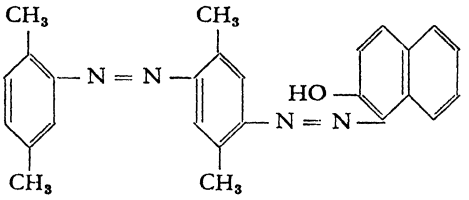


Fig. 42. Patterns of a normal and a hyperlipaemic serum dyed with bromophenol blue (above) and oil red O (below). Automatically scanned patterns and actual strips are shown. (Durrum *et al.* [38]).

one hour in the third wash, the lipid-free sections are again white. The strips may then be cut into 5 mm strips and the dye eluted with 3 ml of 96 % ethanol containing 10 % acetic acid. The absorption is measured with a Beckman spectrophotometer at 595 mμ. Typical pathological patterns are shown in Fig. 43 from Wunderly, Gloor and

Hässig [40]. These patterns confirm again the contention that for most purposes visual examination is adequate for clinical results. For staining with cerol *B* in 50% ethanol see Raynaud *et al.* [94]. A quantitative cholesterol estimation along the paper was carried out by Boyd [101]. See also Ott and Roth [107] and Blöch and Graf [112].



Oil Red O

ESTIMATION OF FIBRINOGEN

Durrum [1], as well as Flynn and de Mayo [8], found that the fibrinogen present in plasma adsorbs strongly at the point of application of the proteins on the paper when barbiturate buffers are employed. It was recently shown however by Berkes and Karas [41] that fibrinogen migrates as in "free" electrophoresis when a phosphate buffer pH 9.2 is used. It thus forms a band between the beta and gamma globulins usually termed the φ component in free electrophoresis. Berkes and Karas examined plasma as well as peritoneal fluid from patients. The fibrinogen content can not be determined quantitatively as the φ component consists of fibrinogen and globulin fractions of the same mobility. The fibrinogen can however never exceed the amount of φ component.

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LYSOZYME

Caselli and Schumacher [96] determined the position of lysozyme in the serum protein pattern. Lysozyme moves towards the cathode and thus forms a band behind the gamma globulin fraction. These authors suggest that the frequently observed tailing of the gamma globulins may be ascribed to the presence of considerable quantities of lysozyme. The detection of lysozyme in human tears was also recorded by Caselli and Schumacher [97].

THE SUBFRACTIONS OF THE SERUM GLOBULINS

For most clinical work, separation into albumin and four globulin fractions (α_1, α_2, β and γ) is carried out and is sufficient for diagnostic purposes.

Wiedermann [42], by employing a suitable technique, achieved the fractionation into seven distinct globulin fractions (α_1, α_2, β_1, β_2, β_3, γ_1 and γ_2) all of which have also been noted in free electrophoresis. To achieve this separation a very thin line of serum is painted on a strip, which is then electrophorised in a moist chamber and weighed down by a heavy glass lid. This lid prevents the oversaturation of the paper with electrolyte by arresting the flow from the electrode vessels owing to its weight. 250 volts were applied for 9 hours at an optimum temperature of 12–16° C. The technique was employed by Wiedermann [43] for following the excretion of proteins in the urine in certain kidney diseases.

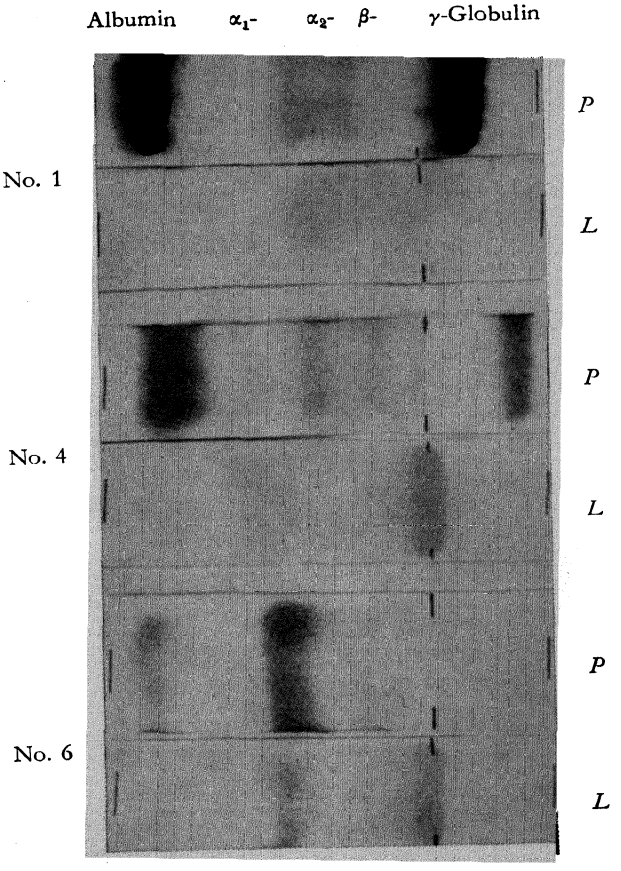

Fig. 43. Comparison of results of paper electrophoresis with protein dyeing (bromophenolblue, marked *P*) and lipoid dyeing (Ciba-blue/Sudan black, marked *L*). No. 1 serum: γ-myeloma; No. 4 serum: Coma hepaticum; No. 6 serum: lipoid nephrosis. (Wunderly *et al.* [40]).

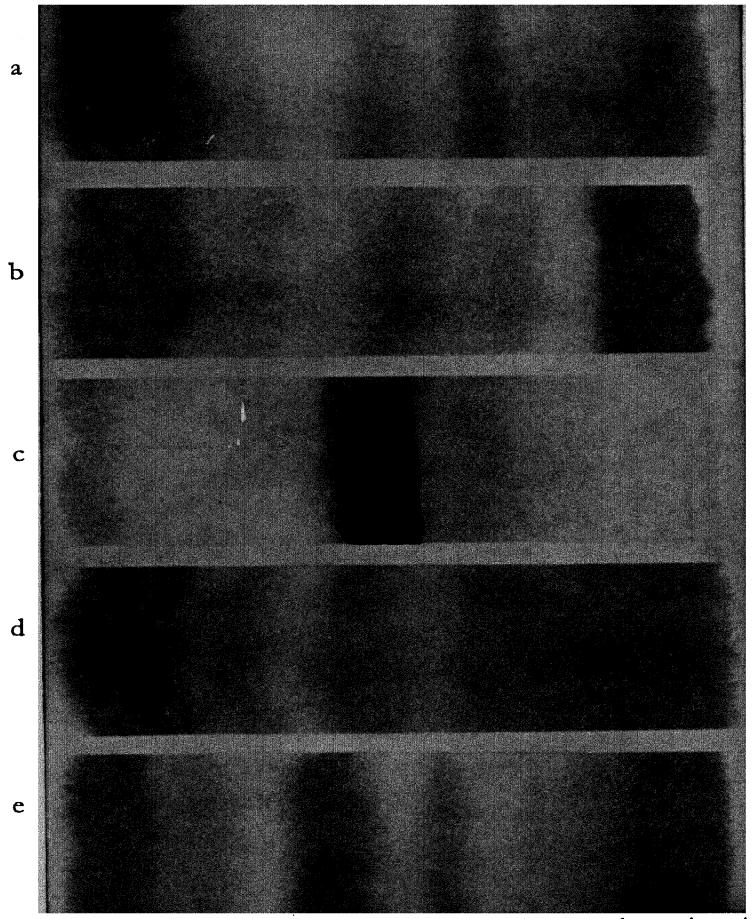

Fig. 44. Typical serum patterns obtained with paper electrophoresis.
1 albumin 2 α_1 globulin 3 α_2 globulin 4 β globulin 5 γ globulin

a normal adult
b multiple myelomatosis (γ globulin much increased but compact)
c nephrotic syndrome (albumin and γ globulin decreased and α_2 globulin increased)
d biliary cirrhosis (increase of γ globulin and of fractions of intermediate mobility between β and γ globulins)
e disseminated lupus erythematosus (γ and α_2 globulins increased and albumin decreased)

SOME CLINICAL RESULTS

It is intended to give only a very brief and incomplete summary of the numerous clinical applications. As will be seen from these, the method is capable of wide application.

Pathological sera

Fig. 44 shows the comparison of various sera by Flynn and de Mayo [8]. Numerous studies of diseases have been published, such as the examination of cancerous sera by Delmon, Blanquet and Babin [44] and myeloma sera by Rice [114].

Analysis of various body fluids in pathological conditions

Cerebrospinal fluid was examined by Everbeck [30], Schneider and Wallenius [46], Caspani [47], Wallenius [48], Bücher *et al.* [49] and Eaton and Gardner [50]; the *aqueous humour of the human eye* by Witmer [51], Wunderly and Cagianut [52] and Wunderly *et al.* [103]. *The proteins of human and animal lenses* were studied by François *et al.*[102], *bladder contents*, *liquid from the pleural cavity* by Jasinsky *et al.* [53] and Wuhrmann and Wünsche [54, 55].

Oedema liquid was examined by Wunderly, Hässig and Lottenbach [56]; *uroprotein* by Suenderhauf and Wunderly[57]. Electrophoretic studies of *bilirubinaemia* were carried out by Wunderly and Reynaud [58]. Red cell stroma was studied by Prankerd *et al.* [106]. Dilute solutions may be concentrated by filtration through collodion (Kutzim [59]). Sper-

matic fluid was examined by Schneider *et al.* [110]. For soluble skin proteins see Spier *et al.* [111].

Haemoglobins

One study of abnormal haemoglobins by Wolfson is mentioned on page 43. Motulsky *et al.* [100] obtained essentially the same results as Wolfson using the ordinary Durrum technique with Whatman 3MM paper pH 8.6 barbital buffer (ionic strength 0.05) with 260–280 volt for 4–4½ hours (optimum time for good separation). Best results were obtained with a solution containing 3± 0.5% haemoglobin. See also Perosa and Bini [104], Singer *et al.* [115] Larson and Rannay [116], Brain [122] and Lehmann [123]. For haemoglobins of *Tetrameres confusa* see Villela and Ribeiro [135, 138].

The sera of various animals

Excellent separations of the globulins in *dog* sera were obtained by Boguth [60]. The globulins yield seven bands which are readily visible. No differences between different races and sexes could be noted, and average results from 20 healthy dogs are quoted.

Sera of the *horse* and the *calf* were separated by Graf and List [6], also by Stöckl and Zacherl [61] and cow sera and several other animal sera by Ganzin, Macheboeuf and Rebeyrotte [62], by Berger [98] and McKinley *et al.* [117]

Paper electrophoresis proved of particular value in experiments with small animals, as samples could be with-

drawn without changing conditions of the experiment, while on the other hand several animals had to be sacrificed for a single separation by the free Tiselius method.

Experiments with *rats*, *mice* and *rabbits* were recorded by Scheiffarth and Berg [63] also by Schneider [64] and Schneider *et al.* [65]. Shark serum was examined by Irisawa and Irisawa [105] and albumins were found absent.

Muscle proteins

Mariani and Toschi [66] separated the muscle proteins on paper with essentially the same sequence as in free electrophoresis, except for actomyosin which stays at the point of application. A buffer of high ionic strength has to be employed for the muscle proteins, and even then they form rather elongated trails. Soluble liver cell proteins were separated in pH 8.6 borate buffer by Adjutantis [95].

Denatured proteins

Several articles by Macheboeuf and Rebeyrotte [67] discuss the detection of protein denaturation on the paper strip, which cannot be detected by free electrophoresis. Serum albumin solution heated for 10 minutes at 80° at pH 7.3, stays in solution and cannot be distinguished by free electrophoresis from undenatured albumin. On paper the denatured protein is strongly adsorbed and forms a long comet instead of a relatively fast-moving round spot.

Serological examination of bands on the paper strip

Wunderly and Hässig [68] were able to demonstrate that a single albumin band on the paper is serologically hetero-

geneous. By cutting up the bands transversely and examining their extracts with antihuman rabbit serum, it could be readily shown that the serological titre of the sections of the bands is in no relation to the quantity of albumin.

Egg white proteins

Egg white albumin from fresh hen eggs yields a pattern, as shown in Fig. 45, which is identical with those obtained

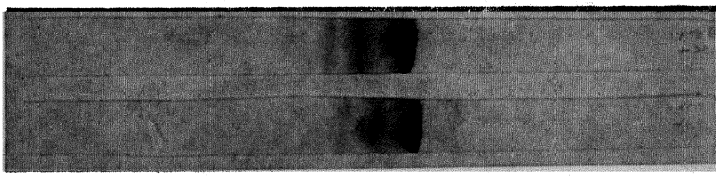

Fig. 45. Separation of the proteins of egg white with *M*/15 phosphate buffers at pH 6.8 (above) and pH 8 (below). (M. Lederer, unpublished; photo University of Queensland).

inside agar (page 184) and by free electrophoresis. Both old solutions and solutions prepared from dehydrated egg albumin, can readily be distinguished by paper electrophoresis from fresh egg white. The amniotic fluid of fertilised hen eggs produces well defined separations, and preliminary work indicates good prospects for the application of this technique in the study of virus-infected eggs as well as in the study of the change of proteins during ripening of an egg. (M. Lederer, unpublished work at the Queensland Institute of Medical Research.) A study of egg white using Tiselius electrophoresis, paper electrophoresis and immunological reactions was published by Kaminski [67].

Ovoglobulin fractions were separated into two bands using veronal buffer at pH 8.3.

Plant proteins

The proteins of the potato tuber were separated into four well-defined bands by Schwarze [70] using the Elphor apparatus with 220 volts for 24 hours. Pfeil and Kanngiesser [71] report the separation into five bands by continuous separation with the Elphor-V apparatus. For plant proteins see also Schneider and Sparmann [128].

Milk proteins

Slater and Van Winkle [119] examined milk proteins. Lindquist [120] used paper electrophoresis in investigations of cheese. See also Aschaffenburg and Drewry [130] and Vandegaer and Miettinen [140].

Snake and insect poisons

One of the first publications on paper electrophoresis deals with the fractionation of the poisons of *Bothrop* species (von Klobusitzky and König [139]).

Wieland and Wirth [21] also Neumann and Habermann [72] showed that snake and bee poisons could be fractionated by paper electrophoresis. Grassmann and Hannig [73] carried out studies with numerous snake and insect poisons. Specific enzymic activity was usually associated with one particular band on the paper. Most separations were carried out at pH 8.6 and the bands revealed with Amidoschwarz 10b or ninhydrin. For work on the flavoproteins in the blood plasma of *Bothrops jararaca* see Villela *et al.* [133].

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Tuberculin constituents

Rhodes and Sorkin [99] reported the separation of tuberculin into a number of well defined spots using phosphate buffer pH 8.3 (ionic strength 0.1) or veronal buffer pH 8.6 with Munktell 20/150 paper, 200 volt for 19 hours at 4°C. Further work was described by Sorkin *et al.* [141], [142]. For separations of Diphtheria toxin see Poulik [118]. For studies on antibodies see Tekman and Ugur [121].

Enzymes

The detection of enzymes on paper was achieved by Wallenfels and Pechmann [74], who used cinematographic film on the paper to reveal proteinases, and starch-iodide for amylases, and by Mills and Smith [75] who used phenolphthalein glucuronide for glucuronidase, phenolphthalein phosphate for phosphatases, and *p*-nitrophenyl butyrate for esterase. Other workers cut the paper up and test the eluate by conventional methods.

There are already numerous papers in which successful concentration and separation of enzymes are recorded. The multiple nature of ox-spleen-β-glucuronidase was demonstrated [75] using the Cremer and Tiselius method and Whatman paper 3 MM. Cytochrome *c* was purified by Paleus [76] using a 0.05 *M* glycine NaOH buffer at pH 9.87 and a phosphate buffer pH 7.0, 50–150 volts for 20–25 hours. The cytochrome is extracted from the paper strips with 0.1 % NH_3. A brown impurity was removed from preparations of beef heart and a yellow impurity from those of

chicken heart. Ferro- and ferricytochromes also separated.

Nikkilä *et al.*[77] examined commercial enzyme preparations. Amylase (non-crystalline pancreatic, Merck), which gave three peaks by free electrophoresis, showed only one active peak when separated on paper, and thus was separated from inactive constituents. Trypsin (non-crystalline pancreatic, Merck) yielded two peaks of activity, probably of chymotrypsins A and B. These separations were effected with the usual 0.05 *M* barbital buffer at pH 8.6 as used for serum proteins and with 250–300 volts for 3–5 hours. For work on cathepsin see Merten *et al.*[136].

Jermyn[78] and Jermyn *et al.*[79] examined the enzymes of *Aspergillus oryzae* and of horse radish both by paper chromatography and paper electrophoresis. Horse radish peroxidase electrophorised with pH 5.7 acetate buffer for 5 hours at 6 v/cm yielded two components, one travelling 1.0 cm towards the anode, the other 1.6 cm towards the cathode; however by paper chromatography four different active fractions could be isolated.

The enzymes of *Aspergillus oryzae* were separated with veronal buffer 0.025 *M* at pH 8.6, yielding two bands with an esterase activity and five peaks with an amylase activity. Further results obtained by paper chromatography and electrophoresis are tabulated in the original paper.

Cholinesterase activity in horse serum was located in the β-globulin-fraction by Togni and Meier[80] using the usual method for serum proteins after Grassmann and Hannig. Levy and Mazia[81] achieved a threefold increase of activity

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per weight in a paper electrophoretic purification of renal alkaline phosphatase with the Kunkel and Tiselius method with barbital buffer at pH 8.7, and 300 volts for 15 hours. Both the enzyme and a protein yielded elongated tracks, which only separated partially. Using the method of Wieland, Heinrich [82] examined pepsin.

Michl and Haberler [132] demonstrated the presence of peroxidases in tobacco by paper electrophoresis. Xanthine dehydrogenase in rat serum was localised by Mitidieri *et al.* [134].

Continuous electrophoresis of enzymes seems to promise isolation of pure enzymes in macro quantities but has so far been relatively little used. Bamann and Tietz [83] employed it for the separation of seed lipase preparations from *Nigella sativa*, which are claimed to be pure from extraneous impurities.

Intestinal alkaline phosphatase was purified and three protein constituents present eliminated by Roche and Bouchilloux [84].

Viruses

A preliminary publication by Gray [85] indicates possibilities in the application of paper electrophoresis to the study and isolation of plant viruses. Using the Durrum method with Whatman No. 1 paper and staining proteins and viruses with bromophenol blue, Gray could differentiate between the proteins of infected and healthy plants.

Radioactive tracer studies with proteins

Of the numerous papers discussing the use of ^{131}I for

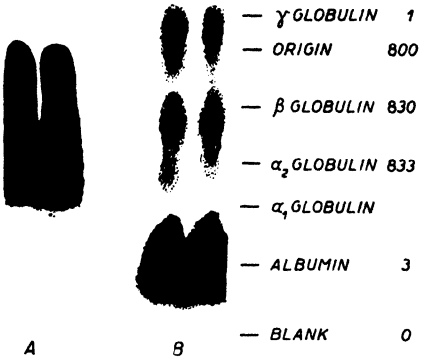

Fig. 46. Distribution of ^{198}Au in the fractions of plasma (Simon [89]).
A radioautograph
B electrophoretic pattern
The figures on the right are the relative GM tube counts.

the study of thyroglobulins in serum, we shall quote Gordon, Gross, O'Connor and Pitt-Rivers [86] also Maurer and Reichenbach [87], Lameijer *et al.* [124] and Gabrieli *et al.* [93]. Either counting techniques or radioautographs (as also used by Durrum [1]) may be used to detect the radioactivity on the paper. Niklas and Maurer [88] also examined the path of methionine marked with ^{35}S and the position of activity in the protein fraction by paper electrophoresis. Simon [89] showed by the use of ^{198}Au, that when colloidal gold is injected into pleural or peritoneal cavities of patients a small quantity is encountered in the circulating blood and this is associeated with the alpha and beta globulin-fractions in the serum as shown in Fig. 46.

Studies with ^{52}Mn, ^{56}, ^{57}Co and ^{131}I in serum and thyroid extracts were published by Horst and Schumacher [90].

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13. Nucleic Acids and Derivatives, and Phosphate Esters

NUCLEIC ACIDS

Kanngiesser [1] examined commercial nucleic acid preparations such as yeast nucleic acid of different ages and gamma globulin nucleic acid.

Separations were obtained in a citrate buffer at a pH between 2 and 3 with 220 volt in 4–6 hours. The nucleic acids were revealed either by dyeing with fuchsin or by dipping into a molybdate solution followed by $SnCl_2$. This work was confirmed by Schümmelfeder and Heyer [2] who showed that both deoxyribose nucleic acid and ribonucleic acid yield three bands at pH 2.5 in a veronal acetate buffer in two hours with 110 volt on S & S 2043a paper. No separation occurs at pH 8.6. The authors suggest that depolymerisation might occur in the pH 2–3 range.

Deimel and Maurer [3] also record separations of ribo- from deoxyribonucleic acid using a veronal sodium buffer at pH 8.6 with 10 volt/cm for 6 hours. Both nucleic acids in this work were marked with ^{32}P and a good separation of three activity curves corresponding to ribonucleic acid (moved 15 cm), deoxyribonucleic acid (moved 20.5 cm) and inorganic orthophosphate (moved 22 cm) was obtained.

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HYDROLYSIS PRODUCTS OF NUCLEIC ACIDS

Several workers have used paper electrophoresis with great success in studies of the degradation products of nucleic acids. Early papers indicating the possibility of nucleotide separation were published by Markham and Smith [4], Davidson and Smellie [5] and Wieland and Bauer [6].

Davidson and Smellie [7], using Whatman 3 MM paper in an apparatus similar to Durrum's with 0.02 *M* citrate buffer at pH 3.5 with 600 volt for 18 hours (length of paper strip 72 cm), separated uridylic, guanylic, adenylic and cytidylic acids (in that order) in quantities up to 300 micrograms. The spots were detected with ultra-violet light. Ribonucleic acids from yeast, liver etc. were examined after alkaline hydrolysis by the method of Schmidt and Thannhauser [8] and quantitative estimations were carried out and found to agree well with determinations subsequent to paper chromatographic separation.

Paper electrophoresis was also found to be very suitable for eliminating radioactive contaminants when working with ^{32}P marked nucleic acids. A second paper by Davidson and Smellie [9] described the separation of nucleotides and the detection of non-nucleotide phosphates (marked with ^{32}P) formed during the degradation.

Dimroth *et al.* [10], using the method of Wieland and Fischer with S & S 2043 b paper, 110 volt at pH 3.1 for 14.5 hours confirm the separation of cytidylic, adenylic, guanylic and uridylic acids in the same order as Davidson

and Smellie [7]. These authors also examined the movement of the *nucleobases* and obtained a good separation of adenine from the other three, which however form a single spot. Jaenicke and Vollbrechtshausen [11] employing a borate buffer at pH 9.2 with 220 volt for 14 hours could realise only a partial separation of the ribosides, except for uridine which moves faster than the rest. Dimroth *et al.* [10] developed a scheme for the examination of nucleic acid degradation products formed by hydrolysis with metal hydroxides. As shown schematically in Fig. 47 borate at

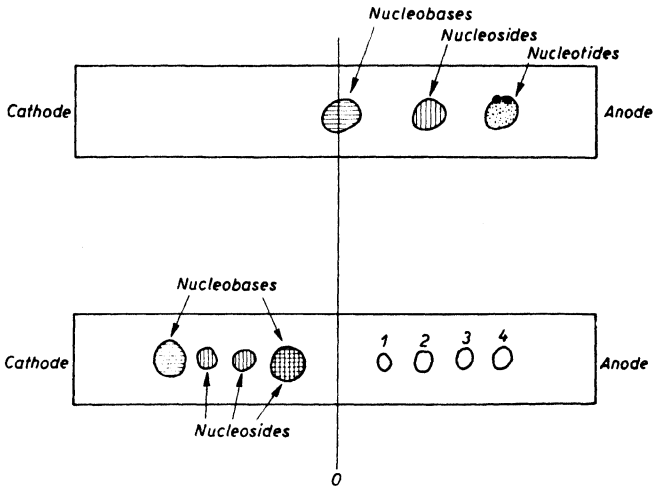

Fig. 47. Separation of nucleobases, nucleosides and nucleotides
Top: pH 9.2, borate buffer
Bottom: pH 3.1, acetate buffer
1 cytidylic acid
2 adenylic acid
3 guanylic acid
4 uridylic acid

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pH 9.2 separates nucleobases, nucleosides and nucleotides into three spots. In a pH 3.1 acetate buffer nucleotides are completely separated from each other and also from the nucleosides and nucleobases.

Burke [25] records the M_G values of purines, pyrimidines, nucleosides and some synthetic pyranoside nucleosides under the same conditions as Foster (see page 78). See Table 20.

Markham and Smith [12] carried out extensive work on the separation of mono-, di- and trinucleotides using a liquid heavier than water as cooling agent for the paper (for apparatus see page 40). Strips of Whatman No. 3 paper 56 cm × 8 cm were employed with 0.05 *M* acetate (also phosphate) buffers and a potential of 1000 volt was applied. Cyclic nucleotides had been obtained by careful alkaline hydrolysis of ribonucleic acid and ordinary and cyclic cytidylic acids could be separated in one hour in phosphate pH 7.4 buffer at 20 volt/cm; the cyclic nucleotides having more OH groups move more slowly. In two other papers [13, 14] accounts are given of the separation of the smaller products of ribonuclease digestion; the mobilities of twenty-three mono-, di- and trinucleotides were tabulated as shown in Table 21. The enzymic breakdown of deoxyribose nucleic acids was also studied by the same authors in another publication [15]; the dinucleoside monophosphate diesters formed moved with the speeds indicated in Table 22.

TABLE 20

M_G VALUES OF PURINES, PYRIMIDINES AND NUCLEOSIDES (BURKE) [85].

Compound	M_G value
Purines and Pyrimidines	
Thymine	0.42–0.59 with a comet
Uracil	0.95
Cytosine	0.0
Adenine	0.35–0.56 with a comet
Ribonucleosides	
Uridine	1.05
Cytidine	0.64
Adenosine	0.60
Guanosine	0.95
Desoxyribonucleosides	
Thymidine	0.51
Desoxycytidine	0.0
Desoxyuridine	0.67
Desoxyadenosine	0.06
Desoxyguanosine	0.59
Synthetic pyranoside nucleosides	
1-D-arabinopyranosylcytosine	0.39
1-D-arabinopyranosyluracil	0.81
1-D-xylopyranosylthymine	0.48
1-D-glucopyranosylthymine	0.53
1-D-galactopyranosyluracil	0.76
1-D-galactopyranosylcytosine	0.40
1-D-glucopyranosyluracil	0.64
1-D-glucopyranosylcytosine	0.15

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SEPARATION OF ATP, ADP AND RELATED SUBSTANCES

Of numerous papers dealing with the separation of ATP and related compounds we shall mention Turba and Enenkel [16] who separated adenine, adenosine, adenosine-5-phosphate and ATP in that order using an acetate buffer of ionic strength 0.1 at pH 4 for three hours with 110 volt (paper: S & S 2043b, 15 cm × 4.5 cm). AMP – ADP – ATP separated in that order in 16 hours under the same conditions.

A detailed paper on the separation of muscle extracts was published by Wollenberger [17] using Munktell 20 paper in the method of Kunkel and Tiselius with a sodium succinate – sodium chloride buffer of ionic strength 0.45 and pH 5.6 with 10 volt/cm and for 165 minutes. Good separations of phosphocreatine, ADP, ATP, adenosine monophosphate, creatine, anserine and carnosine were obtained.

A quantitative technique for the analysis of ATP and ADP preparations was worked out by Wade and Morgan [18]. Whatman No. 3 paper, washed free of Ca and Mg with *N* formic acid, is used. The electrodes are directly attached to the paper as described by Strain (see page 58). Strips 28 cm × 15 cm are used with a pH 3.2 buffer consisting of an aqueous solution of 4.6 % *n*-butyric acid and 0.057% NaOH with 300–400 volt. The spots to be analysed are placed 1.9 cm from the cathode. AMP (adenosine-5-phosphate) travels slowest, then ADP, ATP, PO_4^{---} and pyrophosphate fastest. All spots are completely separated and

TABLE 21

MOBILITIES OF MONO-, DI- AND TRINUCLEOTIDES (MARKHAM AND SMITH [18])

Adenylic, guanylic, cytidylic and uridylic acids are represented by A, G, C and U respectively while their (cyclic) monohydrogen phosphate derivatives are represented by A!, G!, C! and U! respectively.

In a dinucleotide the order in which the nucleotides are written is such that the second nucleotide has a free 3'-phosphate or the equivalent and is linked by its 5'-hydroxyl group to the preceding nucleotide. Thus GU! is the 2':3' cyclic uridylic acid 5'-ester of guanosine 3'-phosphate (the 5'carbon being the only free position for the ester link in the cyclic uridylic acid), while GU is the similar dinucleotide containing uridine 3'-phosphate 5'-ester of uridine 3'-phosphate.

Nucleotide	Mobility in cm/2 hours at pH 3.5 with 20 volt/cm
A	8
G	14
C	6.5
U	16
A!	8
G!	14
C!	7
U!	16
AC!	9
AU!	16
UU!	22
AC	8
AU	16
AG	15
GC!	15
GU!	20
GC	13.5
GU	19.5
UUU!	24
ACC!	6
AAC	13.5
AAU	17
AGU	19

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TABLE 22

MOBILITIES OF DINUCLEOTIDE MONOPHOSPHATE DIESTERS FORMED FROM DEOXYRIBOSE NUCLEIC ACID (SMITH AND MARKHAM [15])

Substance	Mobility in cm/2 hours with 20 volt/cm at pH 3.5
A – p – C	2.0
A – p – T	6.2
G – p – T	9.0
C – p – T	3.9
T – p – T	10.9
C – p – C	0

A = deoxyadenosine
G = deoxyguanosine
C = deoxycytidine
T = thymidine
p = phosphoric acid residue joining two nucleosides.

can be excised for subsequent determination. See also Schild and Maurer [19, 20] for separations for ATP and related compounds, in a pH 8.6 veronal buffer with 200 volt in 3.5 hours.

THIAMINE AND THIAMINE PHOSPHORIC ESTERS

A paper by Siliprandi and Siliprandi [21] describes the separation of thiamine and its mono-, di - and triphosphates by partition and ion exchange chromatography and electrophoresis on paper as well as on starch and paper pulp columns.

For electrophoresis on paper they employ Munktell 20 paper with an acetate buffer at pH 5.44 (ionic strength 0.05) for 7½ hours with a current of 3 mA (the potential is

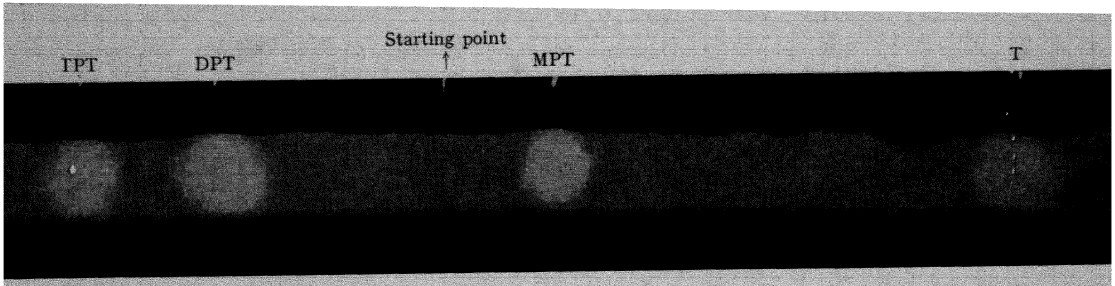

Fig. 48. Separation of a mixture of thiamine phosphoric esters by paper electrophoresis. 50 μg of each compound. Acetate buffer at pH 5.44, μ 0.05. 7.30 hours at a current of 3 mA. Munktell 20 paper.

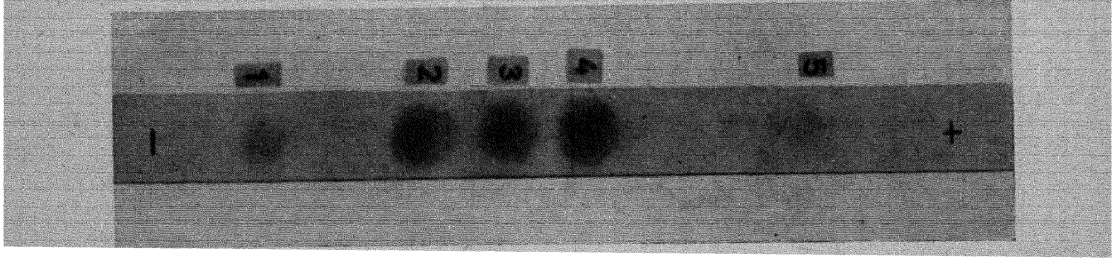

Fig. 49. Separation of a vitamin B_6 compounds by paper electrophoresis (acetate buffer pH 5.1, $\mu = 0.05$, 3.0 mA current during 5 hours). 1 = pyridoxamine; 2 = pyridoxine; 3 = pyridoxal; 4 = pyridoxamine-phosphate; 5 = pyridoxal-phosphate; 25 μg of each compound. The line indicates the origin of the initial spot.

not given). Four well separated spots are obtained: triphosphothiamine moves towards the anode followed by diphosphothiamine. Thiamine and monophosphothiamine move towards the cathode (Fig. 48). See also an earlier paper by Rossi-Fanelli *et al.* [23].

VITAMIN B_2 AND B_6 GROUPS

In another paper Siliprandi, Siliprandi and Lis [22] separate the vitamin B_2 and B_6 groups with acetate pH 5.1 – ionic strength 0.05 buffer (Fig. 49). With 3.5 mA for 12 hours the following order is obtained:

Anode end
pyridoxal phosphate
triphosphopyridine nucleotide
diphosphopyridine nucleotide
flavin adenine dinucleotide
flavin mononucleotide
pyridoxamine mononucleotide
riboflavin
nicotinamide overlapping riboflavin and pyridoxal
pyridoxal
pyridoxine
Cathode end pyridoxamine.

For the estimation of purine alkaloids in, e.g., coffee, tea and cocoa, see Michl and Haberler [24].

Work on ^{32}P labelled hexose phosphate esters was described by Schild and Bottenbruch [26]. For flavins see Cerletti and Siliprandi [27] and Ribeiro *et al.* [28].

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14. Antibiotics

Paper electrophoresis has been used in the search for unknown antibiotics, by Hosoya *et al.* [1] and by King and Doery [2]. The latter used microbiological methods to test for the spots and found that adsorption on the paper and movement of the spots during drying can interfere with the separation. Chloromycetin was always run as reference substance on each strip of paper.

Foster and Ashton [3] record separations of a number of substances related to streptomycin. The mobilities are shown in Table 23. The spots were revealed chemically not microbiologically. Three sprays are suggested: (i) a diacetyl spray, (ii) a naphthoresorcinol spray and (iii) the Elson Morgan reaction. Whatman No. 31 (extra thick) paper was used (24 cm × 70 cm) with a pH 5 acetate buffer (700 ml 0.2 *M* sodium acetate and 300 ml 0.2 *N* acetic acid) in an apparatus after Kunkel and Tiselius.

TABLE 23

MOBILITIES OF STREPTOMYCINS (AFTER FOSTER AND ASHTON [3])

Substance	Mobility $\mu \cdot 10^{-5}$
Streptomycin	22.5
Mannosido streptomycin	19.5
Streptothricin	24.0
Streptidine	24.9
Streptamine	6.3

For the separation of antibiotics of *E. coli* see also page 189.

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15. Triterpenoids

Hashimoto [1] converts triterpenoids to their bisulphates by treating them with pyridine – SO_3. The bisulphates were electrophorised with organic solvents such as butanol: water: acetic acid (5:4:1) or phenol saturated with 0.5% borax solution on Toyo paper 1 cm wide and 40 cm long.

The separated bands were detected by heating to 110° and spraying with $SbCl_3$ in chloroform (after Neher and Wettstein [2]) so that purple or blue spots appear.

Table 24 shows the movement (in mm) of the various triterpenoids. See also Shimano *et al.* [3].

TABLE 24

MOVEMENT OF TRITERPENOIDS

Substance	solvent	
	(1)	(2)
β-Amyrin	51 mm	26 mm
Betulic acid	43	11
Betulin	36	20
Hederagenin	29	5
Lupeol	24	17
Morolic acid	38	23
Oleanolic acid	35	12
Ursolic acid	33	26
Cholesterol	45	15

Solvents: (1) butanol: water: acetic acid 5:4:1 (upper layer), 600 volt, 0.3 mA/cm, 5 h; (2) phenol: 0.5% borax 9:1, 600 volt, 0.01 mA/cm, 3 h.

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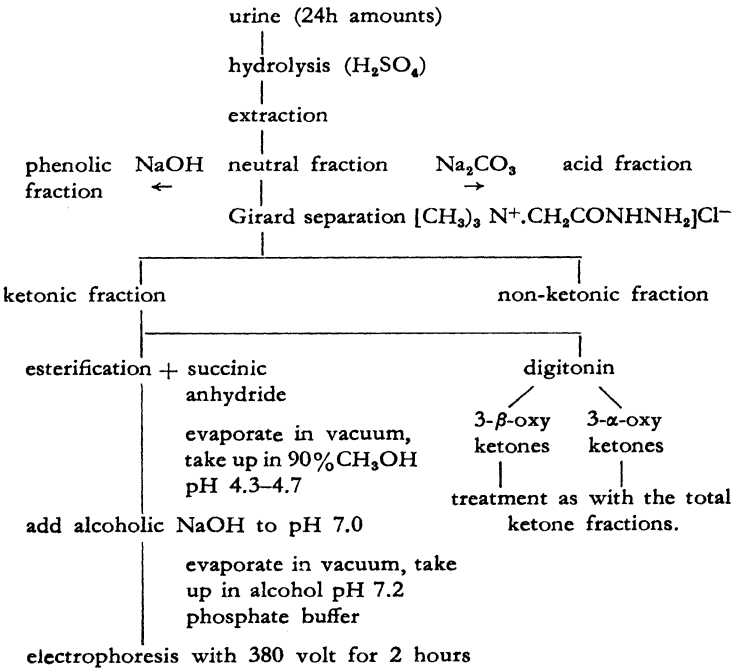
16. Steroids

Voigt and Beckmann [1] describe the separation of the succinic half esters of steroids from urine extracts of normal and pathological cases.

As electrolyte a mixture of a pH 7.2 buffer (4 parts) with methanol (1 part) is used with 380 volt for 2 hours The complete analysis scheme which precedes the electrophoretic separation is given below:



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By spraying the paper with reagents such as phosphoric acid, $SbCl_3$ or anisaldehyde-H_2SO_4 up to five UVL-fluorescent bands may be detected and quantitavively estimated by spectrophotometry.

By electrophoresis of larger quantities it was possible to crystallise the separated bands and indentify them by their melting points. These authors also publish a number of tables showing the quantitative distribution of the various bands in a number of clinical cases. See also McKinley [2].

Steroid conjugates were separated by Levin and Davies [3].

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17. Dyes

Electrophoresis on paper was employed by Evans and Walls [1] for the examination of dyestuff mixtures used in bacteriology. Using the Durrum technique with Whatman No. 1 paper strips 1 cm × 34 cm and 220 volt for two to fifteen hours with 0.1 *N* NH_4OH (pH 11.1), eosin Y was separated into three bands. Wright's stain could be separated (using 0.2 *M* phosphate buffer at pH 7) into a band of eosin and one of methylene blue. The components of eosin did not separate in this buffer.

A mixture of eosin Y and crystal violet was separated in 2.5 hours with 0.02 *M* phosphate buffer at pH 5.7, into an orange eosin band (moved 2 cm), a red eosin band (moved 1.4 cm) and a crystal violet band (no movement). At pH 8.5 in 15 hours the yellow eosin band moves 8.5 cm, while crystal violet stays at the point of application.

Acid fuchsin was separated into numerous fractions by the continuous method of Durrum [2].

A mixture of methyl orange and phenolphthalein was separated by Strain and Sullivan [3]. Bromophenol blue and cresol red (also an impurity) were separated continuously by Grassmann and Hannig [4].

Some acridine dyes were separated with *N*/10 HCl as electrolyte with 8 volt/cm in one-and-a-half hours. Atebrin travels fastest, then follow 5-amino-acridine, acriflavine and proflavine (slowest) [5]. Paul and Durrum [6] employ

References p. 148

organic solvents for the paper electrophoresis of dye mixtures. Oil red O and crystal violet were separated with nitromethane – acetic acid (9 : 1) with 500 or 1000 volt in 15 minutes. In absolute alcohol a mixture of eosin, methyl violet, oil red and alizarine blue was separated with 1000 volt (60 μA) in 50 minutes. See also Mori and Kimura [7] on the separation of food colours, Franglen[8] on sulphonphthaleins and Patti [9] on eosins.

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18. Inorganic Compounds

The separations of inorganic substances by electromigration have already been reviewed by the author [1]. One group of workers in this field [4, 8, 10] insists on the importance of adsorption in the separations obtained and applies the name electrochromatography in preference to electrophoresis (see also Introduction, page 7). The effect of adsorption is undoubtedly large but as the same techniques are applied as in protein and other separations the same terminology can be adhered to.

If one consults the ionic mobility tables on page 15 one gains the impression that the use of electrophoresis is limited to the separations of ions with different charges if the ions migrate as in infinite dilution and without complex formation. The electrolytes employed are however all complexing agents and the separations effected are due to the different degrees of dissociation of the complexes.

SEQUENCES OF IONS

Strong electrolytes

The movement of a number of metallic ions in *N* HCl was examined by Lederer and Ward [2] using the Durrum technique with 70 volt for 1½ to 3 hours. The movement of the ions is slow, owing to the high concentration of HCl, and the ions were divided into cationic, anionic and isoelectric, as shown in Table 25.

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TABLE 25

MOVEMENT OF METAL IONS IN 1 *N* HCl

Metal	Sign of charge	Metal	Sign of charge
Mo^{VI}	+	Fe^{III}	+
As^{III}	+	Co^{II}	+
Cu^{II}	+	Ni^{II}	+
Hg^{II}	—	Sb^{III}	—
Pd^{II}	—	Sn^{II}	+
Pt^{IV}	—	Pb^{II}	+
Au^{III}	—	Bi^{III}	—
		Cd^{II}	0

After these preliminary results Lederer and Ward [3] examined *N*/2 HCl as electrolyte and at the same time improved the separation by sandwiching the paper between glass plates to avoid undue evaporation. In the use of strong electrolytes the technique plays an important part, as the current passed is high and the temperature of the paper considerably raised. Employing 110 volt for 50 minutes (gradient 3.6 volt/cm) metallic ions can be separated into five groups given in Table 26.

TABLE 26

MOVEMENT OF METAL IONS IN *N*/2 HCl IN 50 MINUTES WITH 3.6 VOLT/cm

Anionic		Isoelectric	Cationic	
2.5–1.5 cm	1.5–0.5 cm		0.5–1.5 cm	1.5–2.5 cm
Hg^{II} Pd^{II}	Au^{III} Ir^{IV} Bi^{III} Tl^{III}	Cd^{II}	Mo^{VI} Zr^{IV} Rh^{III} As^{III} Sn^{II} Sb^{III} Pb^{II}	Cr^{III} UO_2^{II} Ti^{IV} Be^{II} Fe^{III} Co^{II} Ni^{II} Al^{III} Cu^{II} Zn^{II} Sr^{II} Ba^{II}

Under the conditions stated, ions in the same group do not separate. With higher potential (10 volt/cm) separations of Co^{II}-Fe^{III} and of Pd^{II}-Hg^{II} were obtained. Commercial rhodium chloride (dark red) yields two bands, and some chromium chloride solutions produce two to three spots which are not well defined, agreeing well with the complex chemistry of Rh^{III} and Cr^{III} chlorides. For the separation of Cr^{III} complexes see [17, 33, 34, 35, 36, 37].

Hydrobromic acid (0.42N = 5%) was also investigated [14] as electrolyte and found to be more efficient than HCl in some separations owing to its greater complexing ability and the resultant larger differences in electrophoretic movement.

The mixtures of cations to be placed on the paper were first evaporated with conc. HBr to ensure formation of the bromo complexes and then made approx. 0.4 N with HBr solution.

The separation of Pt^{IV} and Pd^{II} which is not possible in HCl owing to comet formation of the Pt^{IV} can be readily achieved in HBr. The movement of the ions in the same apparatus as for $N/2$ HCl with 150 volt for 1 hour is as follows:

Cu^{++}	+27 mm	Pt^{++++}	−42 mm	Tl^{+++}	−20 mm
Pb^{++}	−24	Pd^{++}	−53	Zn^{++}	+21
Cd^{++}	−41	Au^{+++}	−15	Co^{++}	+25
Bi^{+++}	−46	As^{+++}	− 4	Ni^{++}	+23
Hg^{++}	−51	Sb^{+++}	−17	Fe^{+++}	+23
		Sn^{++}	− 3	Al^{+++}	+23

If the mixture is applied as a very thin band, the sepa-

ration of Cu, Pb, Cd, Bi, Hg, As, Sb and Sn^{++} is complete, except for As–Sn^{++} which move together.

Lederer and Ward [3] discuss also the movement of anions using 1*N* KCl as electrolyte as shown in Table 27.

Separations of anions using lactic acid as electrolyte were reported by Nagai and Kurata [32].

TABLE 27
THE MOVEMENT OF ANIONS IN 1*N* KCl WITH 3.65 VOLT/cm FOR 50 MINUTES (EXPRESSED IN mm)

Anion	Distance travelled	Anion	Distance travelled
I^-	42	NO_2^-	32
CNS^-	35	$S_2O_3^{--}$	30
$Fe(CN)_6^{---}$	34	BrO_3^-	23
CrO_4^{--}	32	$Fe(CN)_6^{----}$	23
		$Cr_2O_7^{--}$	21

Maki [23, 24] used poly- and meta-phosphates as electrolytes for cations.

For studies with $NaNO_3$, KCNS and tartaric acid as electrolyte, see Yasunaga and Shimomura [13]. For the use of organic solvents for the separation of cations see Maki [19].

Weak electrolytes

Numerous weak electrolytes capable of forming complexes with metal ions have been examined as to their suitability in paper electrophoresis. Most are mentioned later in this chapter. Sato, Norris and Strain [4] employ 0.1 *M* lactic acid extensively and observed the following sequence, the fastest cation being placed first:

Cs + Rb, Tl, Ra + Ba + Sr + Ca, Cd, Co, Ni + Zn, Pb,

Cu, Y + Nd, Ce + Pm + Pr + Eu, Sc, U, Th, Bi, Po.

In this work some ions such as Zr and Nb were applied in a strong mineral acid, and hence their behaviour and position in the series is only valid if the exact conditions are adhered to. For further details see the original paper.

1 % citric acid was investigated by Lederer [5] and yields a series which in parts is identical to that in 0.1 *M* lactic acid. EDTA as complexing agent was studied by Macek and Pribil [26] and Miller *et al.* [27].

SEPARATIONS OF ANALYTICAL AND PREPARATIVE INTEREST

Separation of Cu^{++}, Pb^{++}, Cd^{++}, Bi^{+++} and Hg^{++}

This separation was investigated at various HCl concentrations by Lederer and Ward [3, 6]. An optimum HCl concentration is reached round *N*/2 as shown in Table 28.

TABLE 28

DISTANCES TRAVELLED BY Cu^{++}, Cd^{++}, Bi^{+++} AND Hg^{++} IN 50 MINUTES WITH 3.9 VOLT/cm (EXPRESSED IN mm)

	Concentration of HCl		
	0.1 *N*	0.5 *N*	1.0 *N*
Cu^{II}	+27	+44	+16
Cd^{II}	+14	4	—17
Bi^{III}	— 4	—30	—40
Hg^{II}	—12	—50	—50

The separation may be carried out in 15 minutes by employing a potential of 270 volt as illustrated in Fig. 50. Lead (not shown in this figure) is found on the positive side just separated from cadmium. See also Maki [12].

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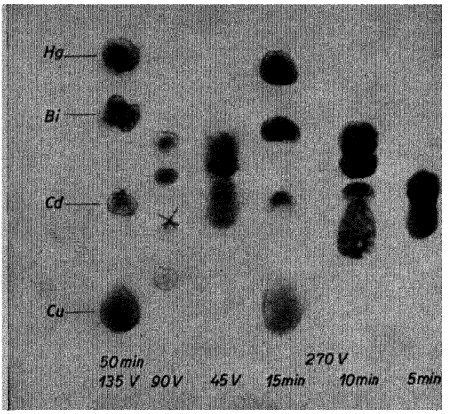

Fig. 50. Separation of Hg, Bi, Cd and Cu, using *N*/2 HCl as electrolyte.

Separation of Ti, V and Mo

The coloured peroxy compounds of these three ions separate with *N*/2 HCl containing 10 % hydrogen peroxide. With 110 volt for 50 minutes Ti forms an orange band 13 mm towards the cathode, V a red band 3 mm towards the cathode and Mo a yellow band 11 mm towards the anode [7].

Separations of As-Sb-Sn, Fe-Co-Ni-Al, Ag-Pb-Hg, etc.

Using the apparatus described on page 58, Strain *et al.* [8] separate numerous metal mixtures as shown in the Figures 51–57. These results illustrate the type of organic complexing agents that may be successfully employed.

Separation of rare earths, Sc and Ac

The cerium group rare earths, scandium and actinium

as well as several other elements were studied [5] using citric acid as electrolyte. The mixture of the rare earths is placed on the paper as citrates and 1 % citric acid employed in the Durrum method with 300 volt for 45 minutes. Table 29 shows the distances travelled under these conditions.

TABLE 29

SEPARATION OF RARE EARTHS AND SOME OTHER METALS

Element	Distance travelled towards cathode (expressed in mm).	Element	Distance travelled towards cathode (in mm).
Sc	5	Th	5
Sm	26	Fe^{III}	5
Nd	30	Al	30
Y	30	Zn	47
Ce^{III}	37	Bi	5
La	46	Pb	ca. 40
Ac	60	Cu	29

The rare earths and the other elements were detected by spraying with ammoniacal alcoholic 8-hydroxyquinoline and viewing under ultra-violet light. Continuous separations were effected with mixtures of Y and La and La and Ac. The method employed was that of Durrum (see page 56 with 355 volt. Fig. 58. shows the separation La-Ac [9]. For the separation Nd-Pm-Sm see Langevin–Joliot and Lederer [29].

Sato *et al.* [10] worked out separations of radioactive rare earths using various organic acids. Strips of Eaton-Dikeman Grade 301 paper (0.03 inches thick, 3–6 feet long and 20 inches wide) were moistened with the electrolyte solution,

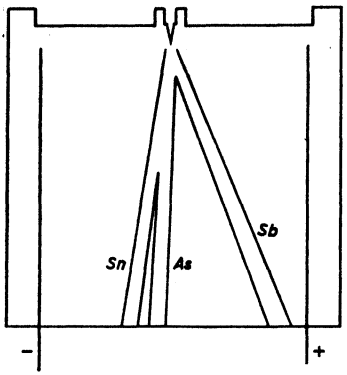

Fig. 51. Separation of stannous, arsenious and antimonious chlorides. Electrolyte, 0.02 *M* lactic acid, 0.02 *M* tartaric acid, and 0.04 *M* DL-alanine. Reagent, H_2S. 300 volt, 95 mA (Strain and Sullivan[8]).

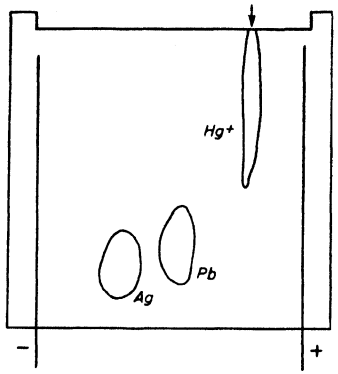

Fig. 52. Discontinuous separation of mercurous, lead and silver nitrates, each 0.05 *M* in 1 *M* HNO_3 (0.01 ml). Electrolyte, 0.1 *M* lactic acid (60 ml). Reagent, H_2S. 250 volts, 100 mA, 20 minutes.

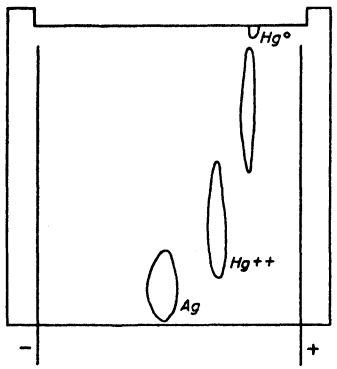

Fig. 53. Discontinuous separation of mercurous, lead and silver nitrates, each 0.05 *M* in 1 *M* HNO_3 (0.01 ml). Electrolyte, 0.008 *M* citric acid in 4 *M* NH_4OH (60 ml). Reagent, H_2S. 160 to 170 volts, 100 mA, 20 minutes.

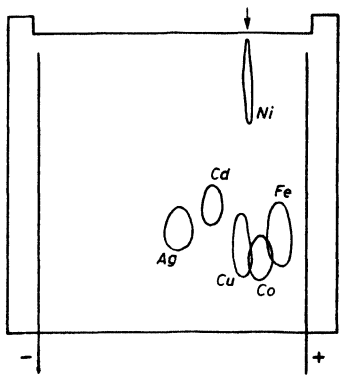

Fig. 54. Discontinuous separation of nickel, ferric, cobalt, copper, cadmium and silver nitrates, each 0.05 *M* in 0.1 *M* tartaric acid (0.01 ml). Electrolyte, 0.01 *M* ammonium tartrate, ca. 0.005 *M* dimethylglyoxime in 4 *M* NH_4OH (60 ml). Reagent, H_2S. 160 volts, 95 to 100 mA, 20 minutes.

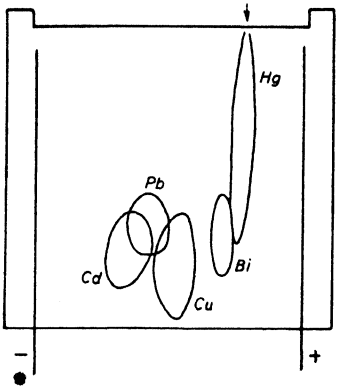

Fig. 55. Discontinuous separation of mercuric, bismuth, copper, lead and cadmium nitrates, each 0.05 *M* in 1 *M* HNO_3 (0.01 ml). Electrolyte, 0.1 *M* lactic acid (60 ml). Reagent, diphenyl-carbazide (Ag), diphenylthiocarbazone (Cd), dithio-oxamide (Cu), dithio-oxamide plus NH_4OH (Pb), Na_2S (Bi). 250 volts, 100 mA, 20 minutes.

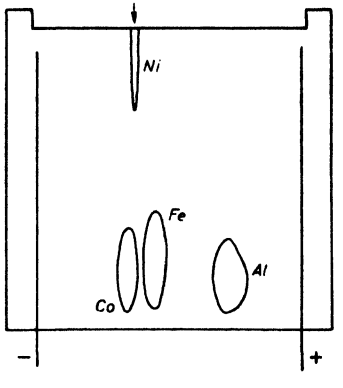

Fig. 56. Discontinuous separation of nickel, cobalt, ferric and aluminium nitrates, each metal 0.005 *M* in 0.01 *M* tartaric acid and 0.005 *M* dimethylglyoxime (0.025 ml). Electrolyte, 0.01 *M* tartartic acid and 0.005 *M* dimethylglyoxime in 4 *M* NH_4OH (60 ml). Reagent, dithio-oxamide, aluminon in 50% acetic acid. 150 volts, 100 mA, 20 minutes.

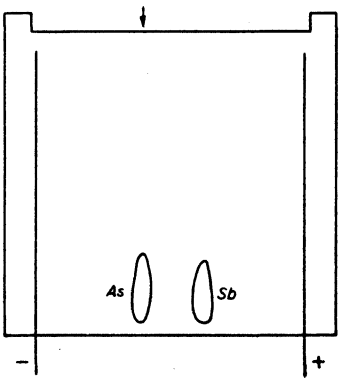

Fig. 57. Discontinuous separation of arsenious and antimonious chlorides, each 0.01 *M* (0.05 ml). Solution and electrolyte, 0.4 *M* DL-alanine in 0.1 *M* lactic acid. Reagent, H_2S. 300 volts, 100 mA, 20 minutes.

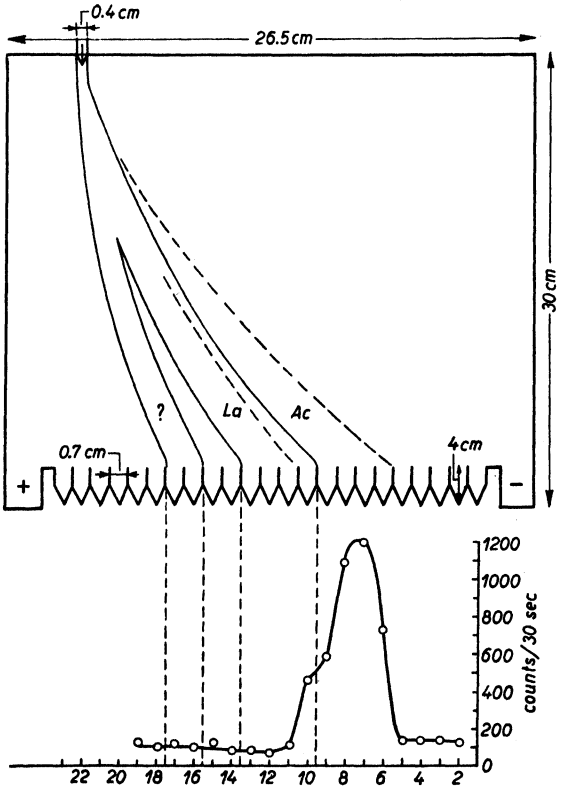

Fig. 58. Separation of lanthanum and actinium. The tracks visible with hydroxyquinoline under ultra-violet light are drawn on the paper sheet. Actinium was detected by the activity of the fractions, as shown in the graph below the diagram of the paper sheet.

and 50 μl of the mixture (about 0.3 microcurie of each activity) placed on the paper. The paper was encased in a polyethylene sheet and its ends allowed to dip into vessels holding the electrolyte solution. A potential of 5 volt/cm

was applied for 1–2 days. The ions were located photographically. Fig. 59 shows the separations obtained with various concentrations of lactic acid and with a mixture of tartaric acid and ammonium tartrate. The following pairs of ions were also separated: Nd-Pm, Ce-Pr, Ba-La and Sr-Y, using 0.1 *M* lactic acid as electrolyte.

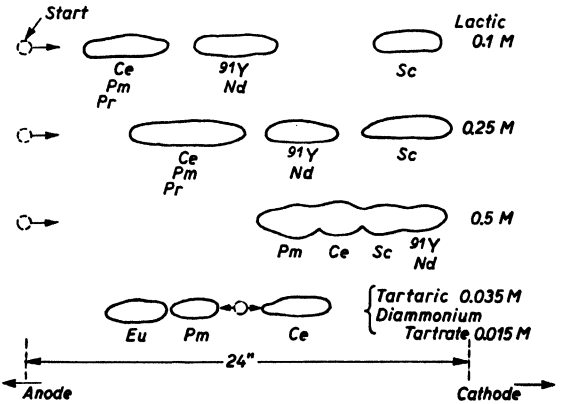

Fig. 59. Migration of rare earths in lactic acid (24 hr.) and in tartrate solution (48 hr.) (Sato *et al.* [10]).

Separation of fission products

According to the data available, tracer amounts of isotopes can be considered as migrating with the same speeds as macroquantities. Strain *et al.* [4, 8, 10] discussed in a number of publications the separation of mixtures obtained from uranium fission. The continuous method seems very suitable for such separations, as little manipulation would be required. Sato *et al.* [4] described the con-

tinuous separation of $^{95}Nb+^{95}Zr$, ^{233}U, ^{90}Y, ^{90}Sr and ^{137}Cs. *Nb and Ta* were separated by Bruninx *et al.* [22].

Separation of condensed phosphates

Sansoni and Klement [11] report the separation of divers mixtures of phosphate ions, using a borate buffer at pH 10 with 110 volt for 3 hours. Illustrations of the complete separations of the following are given:

(i) ortho-, di-, and triphosphates;

(ii) trimeta-, triphosphate-Graham's salt;

(iii) technical sodium tripolyphosphates yielding usually two well-separated bands.

Separation of SO_4^{--}–PO_4^{---} and selenite–tellurite. Both these mixtures separate in *N*/2 HCl as electrolyte [15]. Sulphate moves as an anion while orthophosphate remains on the point of application. Tellurite moves as a cation and separates completely from selenite (no movement) in two hours with 150 volt.

Alkali metals. Separations of Li-Na-Rb-Mg with NH_4OH as electrolyte and 225 volt or 500 volt were described by Harasawa and Sakamoto [16].

More detailed studies of the alkali metals were published by Schier [20] and Evans and Strain [30].

Halides were separated by Ohara and Nagai [18] in lactic acid with 300 volt; *sulphite and thiosulphate* in pH 7 phosphate buffer [21]. For seleno- and telluro-thionates see Wood [31].

Quantitative results by measuring the lengths of the

bands were recorded by Maki[25] for Al, Ba, Co $(NH_3)_6^{+++}$ and Fe $(CN)_6^{---}$

Isotope separation

Using high potentials Bonnin *et al.*[28] achieved partial separations of ^{22}Na and ^{24}Na.

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19. Physico-chemical Studies

As pointed out in Chapter 3, it is possible to measure the isoelectric point of a substance if the corrections suggested by Kunkel and Tiselius are taken into consideration. Fig. 60 shows the determination of the isoelectric point of human serum albumin as measured by Kunkel and Tiselius [1]. On the same graph are the mobilities obtained by the free electrophoretic method and the movement of dextran used to correct for electro-osmosis.

Numerous workers, mentioned below, have published measurements of isoelectric points without correcting for

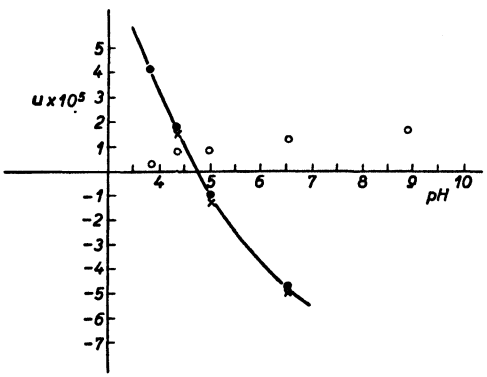

Fig. 60. Determination of the isoelectric point ot human serum albumin (Kunkel and Tiselius [1]).
● mobility of serum albumin on paper
O mobility of dextran on paper
× mobility of serum albumin in free electrophoresis.

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electro-osmotic movement. Their results, especially when dealing with previously unknown proteins, are still of value, since comparison under exactly the same conditions (ionic strength, type of apparatus, voltage etc.) will yield comparative results and facilitate the identification of new substances. It is proposed here, to avoid confusion, to specify in each case whether the isoelectric point is corrected or not as is also done with melting point determinations.

Schwarz[2] was one of the first to determine the isoelectric point of a protein (horse serum albumin) by filter paper

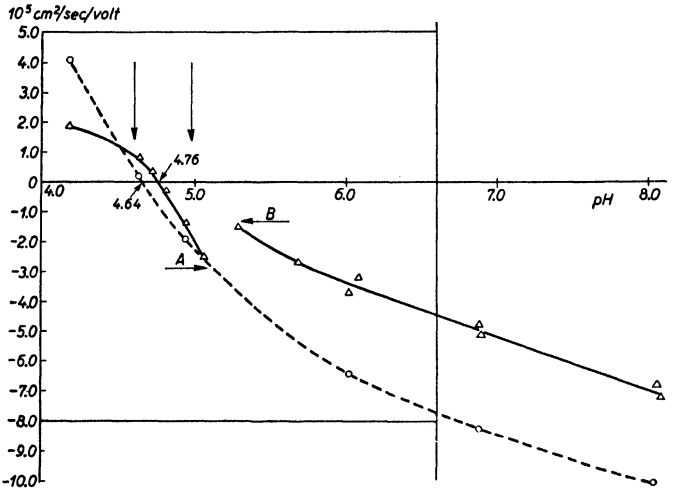

Fig. 61. Determination of the isoelectric point of horse serum albumin by Schwarz[2].
Broken line represents a portion of the curve obtained by Tiselius (*Biochem. J.* 31 (1937) 1464) using free electrophoresis, and corrected for temperature.
A acetate buffers
B phosphate buffers

electrophoresis. Although no corrections were made, the isoelectric point agrees well with values previously obtained by free electrophoresis as shown in Fig. 61. However the break in the curve is due to a change from acetate to phosphate buffer, a change which will certainly affect electro-osmotic flow. Several papers by McDonald *et al.* [3, 4, 5] also deal with the measurement of mobilities and isoelectric points without adequate corrections and McDonald claims that the "data reported indicate that essentially the same isoelectric points are obtained by ionography as are obtained by other methods". One piece of evidence proposed is that the same isoelectric points were obtained for crystalline bovine serum albumin when run on paper and when run on woven glass fibre ribbon. With such arguments it must be remembered that interference may be minimal in some apparatuses, but is very considerable in others such as in the Durrum method, where spots travel also under the influence of the liquid movement due to evaporation during electrophoresis. Thus, unless the type of apparatus and all conditions are stated, uncorrected isoelectric points should still be treated with suspicion.

Interesting work on the larval blood proteins of *Drosophila hydei* was recorded by Wunderly and Gloor [6] as shown in Fig. 62. It is evident that no other method of isoelectric point determination now known could have been employed in such a problem where only minimal quantities can be used. Even here 400 larvae had to be prepared for each single electropherogram.

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Inorganic compounds were studied by various workers. Kraus and Smith [7] used paper electrophoresis (values uncorrected) for demonstrating the change of charge on the chloromercurate complex with change of chloride concentration in the electrolyte.

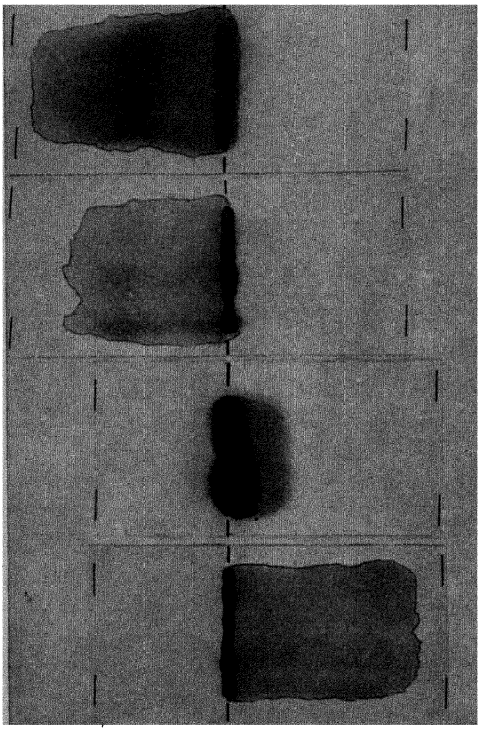

Fig. 62. Determination of the isoelectric point of the larval blood proteins of *Drosophila hydei*. pH range 8.8. to 6.2 (Wunderly and Gloor [6]).

The isoelectric point of $CdCl_2$ was found by Lederer and Ward [8] to be in the region of 0.5 *N* HCl. Below this concentration Cd^{II} travels as a cation and above this concentration as an anion. Lederer [8, 9] also examined the movement of Fe^{III} in thiocyanate solutions with various concentrations of alcohol. In aqueous KCNS, Fe^{III} forms a red cationic complex, while in 66 % alcohol a complex of the same red colour moves anionically. The movement of Po^{IV} in dilute HCl solutions was also examined; Po travels as an anion in *N*/10, *N*/20 and *N*/40 HCl [10].

An attempt at measuring the mobilities of SO_4^{--}, Cu^{++} and Ni^{++} was also made by McDonald *et al.* [11]

Protein-invert soap interactions were studied by paper electrophoresis by Jerchel *et al.* [12, 13]. Their results indicate a complete change of charge with certain invert soaps. Such work should find many applications in surface studies of proteins. For studies of dye-binding ability of proteins see Roth and Kallee [14].

The ionic state of ^{91}Y in human serum was examined by Zilversmit and Hood [15].

Reference p. 168

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20. Electrophoresis in Packed Columns

In this chapter methods of historical interest will not be reviewed. Modern methods suitable for accurate analytical and preparative work were developed mainly in the laboratory of Tiselius.

With these techniques four steps are always required: (i) the filling of the tube with porous material, (ii) the loading of the column with the electrolyte and sample to be separated, (iii) the actual electrophoresis, and finally (iv) the elution of the fractions and their collection in separate receivers.

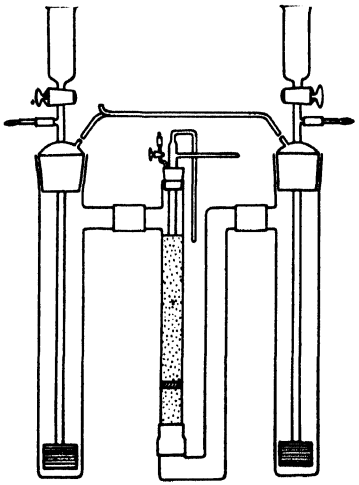

Fig. 63. Principle of apparatus. The two horizontal tubes make it possible for the electro-osmotic flow to develop freely.

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ELECTROPHORESIS IN A GLASS POWDER COLUMN

A preliminary report on the use of glass powder columns by Haglund and Tiselius [1] gives the detailed preparation of glass powder of uniform particle size. The apparatus used (shown in Fig. 63) has an internal cooling arrangement. The sample is placed on the column by applying suction with a syringe driven by a synchronous motor, while the column is immersed first in the solution to be analysed and then in the pure electrolyte. Thus a zone of desired width is introduced into the column as depicted in Fig. 64. Several preliminary experiments with indicators and coloured proteins, using phosphate buffer at pH 7.6 and ionic strength 0.025, yielded satisfactory results. The elution was carried out by using a fraction collector as for chromato-

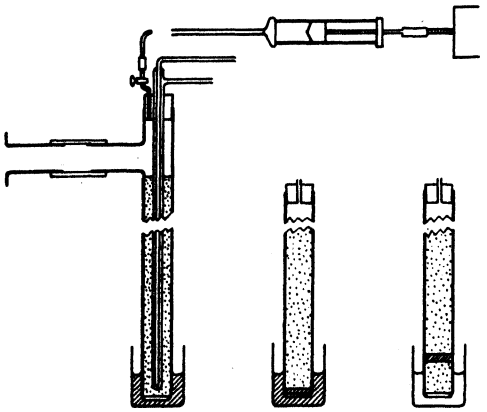

Fig. 64. Filling the apparatus.

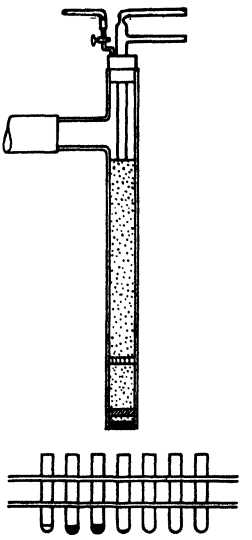

Fig. 65. Sampling after the electrophoretic separation (Figs. 63, 64 and 65 from Haglund and Tiselius [1]).

graphic elution. (Fig. 65). The authors state that much more work is necessary to make this apparatus a good analytical instruments. See also Bradish and Smart [7].

ELECTROPHORESIS IN STARCH COLUMNS

Flodin and Porath [2] point out that to minimise adsorption on glass beads the particles must be relatively large (0.1 mm diameter), which is undesirable since it makes the control of the displacement of the liquid in the column difficult. They worked out a method employing starch, although positively charged proteins did show some adsorption on this medium.

The apparatus used was essentially the same as that employed by Haglund and Tiselius [1], with a tube 50 cm × 3 cm and without the central cooling tube. The column was packed with ordinary potato starch, which was purified by repeated washing with buffer solution. The starch varies somewhat from batch to batch and it is recommended to test the packed column by eluting a suitably coloured zone.

The mixture to be analysed is placed on top of the carefully prepared column just when the buffer has run down to the surface of the starch. This may be done dropwise from a pipette or syringe. When all the solution has been added the column is again perfused with buffer till the zone to be analysed is sufficiently displaced down the column.

Electrophoresis was carried out inside a thermostat. For proteins, veronal buffer of pH 8.6, ionic strength 0.05 with 700 volts (25 mA) was used for 15–20 hours.

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Elution was carried out as with a glass powder column with a flow rate of about 10 ml per hour. Generally 50 fractions of 2 ml were taken. Fig. 66 shows the separation of proteins of normal human plasma with phycoerythrin added.

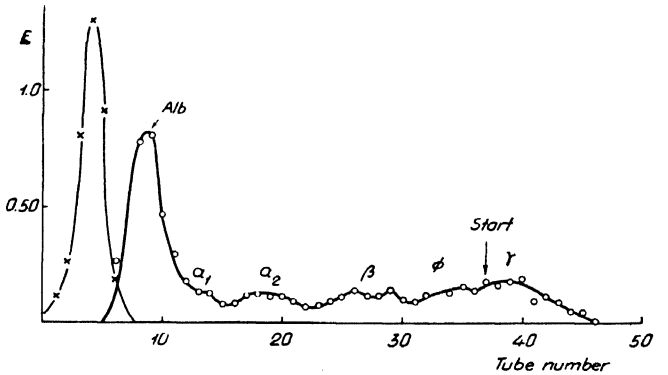

Fig. 66. Distribution curves obtained from an experiment with normal human plasma with phycoerythrin added. Veronal buffer pH 8.6, $\mu = 0.1$, 18 hours with 30 mA. The distribution of the phycoerythrin was measured spectrophotometrically at 535 mμ. The plasma protein was determined by the modified Folin reaction (Flodin and Porath [2]).

ELECTROPHORESIS IN A CELLULOSE COLUMN

The method here described was worked out by Flodin and employed by Siliprandi and Siliprandi [3] for the separation of thiamine phosphoric esters which do not separate well on starch columns, owing to adsorption of the positively charged fractions:

Whatman ashless powdered cellulose was suspended in acetate buffer (pH 5.44 and ionic strength 0.05). After complete exhaustion of the air by evacuation, the slurry

was poured with pressure into a glass electrophoretic column (50 cm × 3 cm).

35 mg of the thiamine ester mixture, dissolved in 2.5 ml of buffer, were placed on the top of the column. The current (30 mA) was applied for 15 hours after 70 ml of the solvent had been allowed to flow down (dead volume of the column being 150 ml). At the completion of the run the column was disconnected from the electrode vessels and the elution was carried out with the acetate buffer. Fractions of 3 ml each, using a flow rate of 25 ml per hour, were taken by means of a fraction collector and analysed as usual.

Quantitative recoveries (100 ± 7 % on the average) were obtained for each component from the integration of the elution curves. A model of a column electrophoresis apparatus is being produced by the LKB Produkter [9].

Extensive work in the amino acid and peptide field was reported by Porath [15].

ELECTROPHORESIS IN SLABS OF CELLULOSE OR STARCH

Kunkel criticized the use of columns in electrophoresis. He points out that adsorption occurs to some extent on all media for packing the column and the elution step, which cannot be avoided, tends to enhance distortion of the separated bands.

A method employing slabs of starch, in analogy to gel electrophoresis, was described by Kunkel and Slater [4], and since the bands can be located in ultra-violet light, or by

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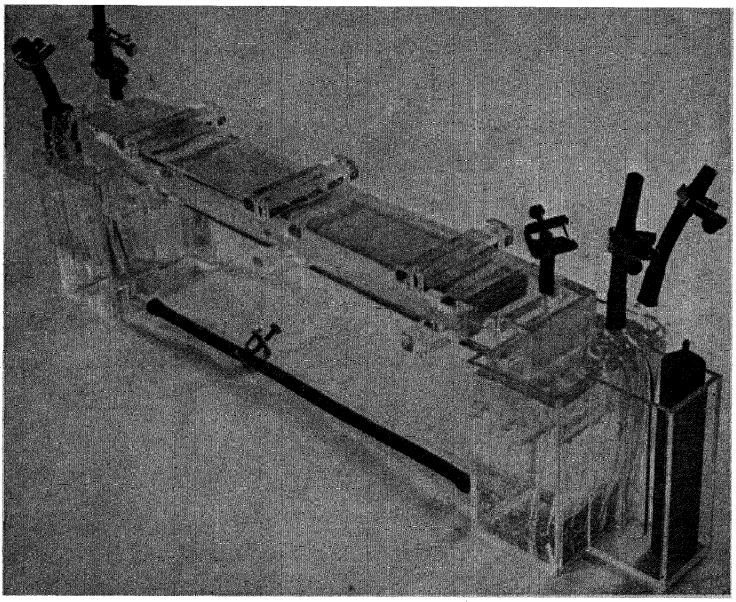

Fig. 67. Apparatus for separation in slabs of starch (after Larson *et al.*[6]).

printing, each fraction can be isolated by cutting out the appropriate section of the slab. The technique is described in detail by Kunkel [5] both for starch and cellulose and, as it is essentially the same as for gels, will not be given here. Fig. 67 shows a set-up for electrophoresis in slabs of starch as used by Larson *et al.* [6].

See also Gordon and McFarlane [11]. For the isolation of ^{35}S labelled albumin see Ulrich *et al.* [8]. A study of serum lipoproteins labelled with ^{32}P and employing this technique was published by Kunkel and Bearn [10].

Smithies [12] has shown recently that electrokinetic ultrafiltration inside starch produces a more complex electrophoresis pattern than paper electrophoresis. Smithies and Poulik [13] obtained numerous subfractions of the globulins by separation on paper and then on starch.

Cetini [14] studied extensively the separation of inorganic ions by electrophoresis inside slabs of starch.

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21. Electrophoresis Inside Gels

As pointed out in the introductory chapters, electrophoresis inside gels was one of the first methods used to illustrate ionic movement in an electric field. After several early separations both of inorganic and organic materials in tubes filled with agar jelly, no extensive application was found for this analytical method till Consden, Gordon and Martin [1] employed an improved technique using initially silica gel in conjunction with paper partition chromatography for the analysis of amino acid and peptide mixtures from wool proteins.

Silica gel has the advantage of containing no organic matter which would interfere with the recovery of pure amino acids, but has the disadvantage of adsorbing proteins to an extent that makes their separation impossible. Agar jelly allows the movement of proteins, but makes isolation of pure proteins difficult owing to the presence of small organic molecules which accompany the proteins on extraction.

Potato starch has been employed as a medium for electrophoretic methods and seems to offer advantages over both silica and agar gels. Its use is discussed in the chapter on electrophoresis in packed columns.

ELECTROPHORESIS IN SILICA JELLY

The technique of Consden, Gordon and Martin [1] will

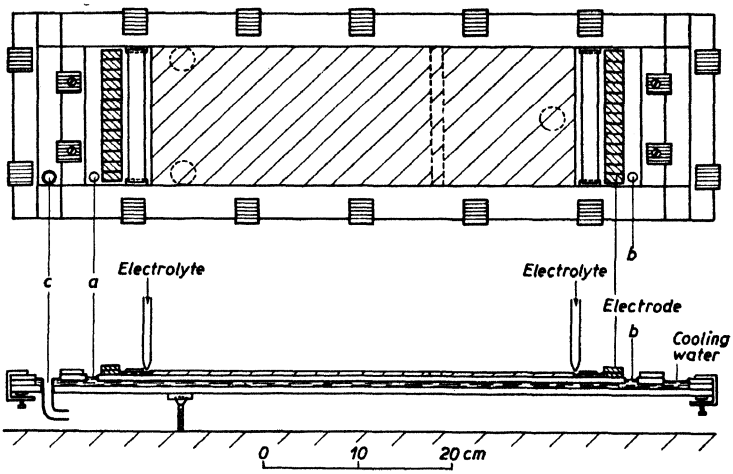

Fig. 68. Plan and cross-section of apparatus used by Consden, Gordon and Martin [1].

be described, as these authors have given detailed instructions for the preparation of silica gel and numerous separations have been carried out with their apparatus.

The trough

This is constructed of $\frac{1}{4}$ inch plate glass. The sides and ends consist of strips of such glass smeared with vaseline and clamped in place. The trough rests on a similar but longer trough, through which water may be circulated to provide cooling. The plan and cross-section with dimensions are shown in Fig. 68. The glass is bored at *a*, *b* and *c* to provide exit holes for the electrode perfusing solutions and for the cooling water. The tube which carries

away the water at *c* protrudes $\frac{1}{8}$ inch above the bottom of the cooling trough. This is necessary to maintain the proper water level in this trough.

Electrode perfusion solution supply

The electrodes are relatively close to the gel and to avoid contamination with electrolysis products, they are perfused with a buffer. A suitable rate of drip may be maintained by syphoning the solutions through long capillary tubes connected to Mariotte bottles. Glass bars, resting on small pieces of microscope slides, are placed in front of the electrodes as shown in Fig. 68. These direct the flow of the electrolyte away from the jelly, thus preventing undesirable ions, produced by the electrodes, from reaching the jelly.

Method of separation for short runs (e.g. separations of amino acids from wool into acid, basic and neutral fractions)

Commercial water glass was diluted approximately twice with water and filtered to give a solution 4.3 *N* with respect to alkali. 28 ml of this solution were diluted to 350 ml with water and 60 ml of approximately 1.6 *M* H_3PO_4 solution added until the pH was just above 7 (blue-green to bromothymol blue). This solution was poured into the trough and allowed to set. The ends of the jelly were cut away to provide compartments for the electrodes. A gutter, 1 cm wide, was now cut out, 23 cm from the anode and on the anode side of the middle to compensate for electro-

endosmosis. The hydrolysate was a solution containing 5 mg N/ml obtained by refluxing wool with 6 *N* HCl for 24 hours, removing excess HCl *in vacuo* and diluting with water. The hydrolysate (1 ml) was made up to 5 ml with sufficient of the above silicate and H_3PO_4 solutions to give the same Na concentration and pH as that of the main jelly. The gutter was filled to the brim with this solution, which then set in about 5 minutes. After the electrodes had been put in place, the perfusion was started. A solution of Na_3PO_4 and Na_2HPO_4 which was grey-blue to thymol blue and *N*/3 with respect to Na, was used for the anode compartment. The cathode perfusion liquor was *N*/8 NaH_2PO_4 brought to pH 6.5 (green to bromothymol blue) with NaOH. Next a potential of 68 volt was applied. which produced a current of 90 mA. After ½ hour the voltage was raised to 190 volt giving a current of 430 mA At this point the cooling water was turned on. After a further 250 minutes the current was stopped and a print was taken by laying a strip of dry filter paper along the length of the jelly, allowing it to become saturated, removing and drying. The strip was sprayed with ninhydrin solution and again dried for 5 minutes at 100° C. Three purple bands appeared, and one 3.5 cm wide had moved 3 cm towards the anode. The second band was 2.7 cm wide and had moved 4 cm towards the cathode. The trailing edge of this band appeared yellow perhaps owing to proline, which always gives a yellow colour with ninhydrin. The third band was 1.8 cm wide and had moved

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7.8 cm towards the cathode. By reference to the print, the jelly was cut into three sections which were each thoroughly mixed with 10 g of paper powder in beakers (the paper powder is Whatman accelerator powder washed with HCl, dried and passed through a 30 mesh sieve).

After standing overnight *in vacuo*, the dry powders were tightly packed into 1.5 cm diameter glass tubes and extracted chromatographically with about 30 ml of water. The solutions were reduced to small volume *in vacuo*, cooled in ice water and filtered from most of the sodium phosphate. The fractions so obtained were tested by paper chromatography. The cathode bands contained only lysine and arginine, the centre band only neutral amino acids.

In the same paper two other methods of electrophoresis are described for separations requiring a long run and for volatile electrolytes.

For a long run, as well as for volatile electrolytes, a glass cover is placed over the gel to prevent surface drying and cracking. Glutamic and aspartic acids were separated in 26 hours with 270 volt and 150 mA at pH 7; also lysine and histidine at pH 6.6. For the separation of glycine and glycylglycine *N*/2 ammonium carbonate is used as electrolyte with 170 volt in 41 hours and 120 mA. In this separation two glycine bands were detected and their existence is explained by the uneven movement of liquid at the glass lid – jelly surface.

The peptides derived from Gramicidin S were separated

by Consden, Gordon, Martin and Synge [2] using silica gel strengthened with paper pulp and 0.1 *M* ammonium acetate as electrolyte. Consden and Gordon [3, 4] separated peptides of cystine in partial acid hydrolysates of wool. Acidic peptides from wool were separated by Consden, Gordon and Martin [5]. The type of separation obtained is shown in Fig. 69.

ELECTROPHORESIS IN AGAR GEL

A thorough study evolving a preparative method by electrophoresis in agar was made by Gordon *et al.* [6]. Their method employs a trough similar to that used for silica gel and the agar was prepared as follows:

5 grams of agar (Difco USP) were added to 500 ml of buffer solution at 80° C and dissolved by further heating. Next, the solution was cooled to 50–55° C and poured into a clean trough. In this work a glass lid was always used. This was placed in position before the jelly was set, care being taken to avoid air bubbles becoming included in the jelly. After setting, the ends of the jelly were cut out, leaving a slab 47 cm long and approximately 0.5 cm thick. The location of the gutter depended on the direction towards which migration was expected. Jellies thus prepared were found non-homogeneous in the electric field; a UVL absorbing substance moving to the anode. This could be removed by a preliminary electrophoresis for 12–14 hours. The protein was introduced into the gutter in the following manner: Salt-containing solutions were

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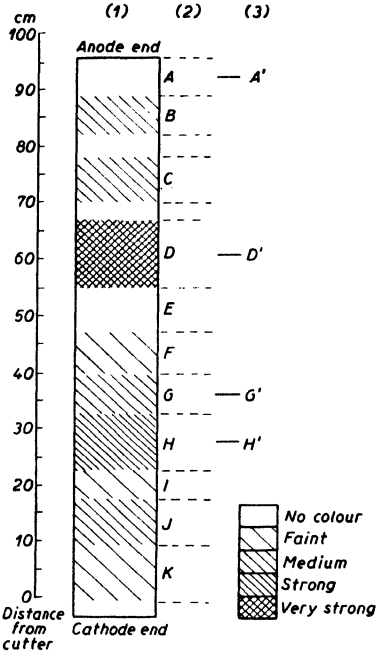

Fig. 69. Ionophoresis of partial hydrolysate of wool (Consden, Gordon and Martin [5]).

(1) Bands on ninhydrin print
(2) Peptides and amino acids comprising bands
(3) Control experiments, movement of synthetic peptides

A dipeptides of aspartic and glutamic acids
B glutamylglutamic acid
C aspartic acid
D glutamic acid
E tripeptides containing two residues of dicarboxylic amino acids
F as *E* and also dipeptides of dicarboxylic amino acids with lower amino acids
G dipeptides, e.g. Ser.Asp, Ser.Glu, Gly.Glu, Ala.Glu, Glu.Ala, Asp.Leu, Glu.Leu
H dipeptides, e.g. Asp.Val, Val.Glu, Leu.Asp, Leu.Glu, Tyr.Glu, Glu.Tyr.
I dipeptides, e.g. Glu.Tyr, Tyr.Glu, Glu.(Cys-), (Cys-).Glu, tripeptides containing one dicarboxylic amino acid residue
J tripeptides containing one dicarboxylic amino acid residue
K not investigated

A' glutamylglutamic acid
D' glutamic acid
G' alanylglutamic acid
H' leucylglutamic acid

dialysed against the buffer. The minimum amount for ultra-violet evaluation or N estimation is about 100 mg of protein. Substances which are not denatured by heating to 40–50° C were mixed with an equal volume of 2 % agar at the maximum permissible temperature either in the gutter or immediately before transfer. Heat-unstable substances were homogenised directly in the gutter with potato starch. Alternatively, 2 % agar was cooled to room temperature with constant careful stirring until a liquid paste free of air bubbles was formed. This was mixed with an equal volume of the protein solution and transferred into the gutter by means of a pipette with a wide orifice. If required, several shorter gutters were cut out side by side. Usually 200 volt (3.4 volt/cm) were applied. The bands were detected after the separation was completed, by printing on paper and spraying the paper with 0.1–0.2% Pauly reagent in N Na_2CO_3, or a band 3 cm wide was cut along the length of the slab and each 1 cm of this introduced into the absorption cell of a Beckmann spectrophotometer, taking readings as a rule at 280–290 mμ. A third method of detection of the bands was a Kjeldahl nitrogen determination on each cm of the jelly. Electroosmosis was determined by the movement of glucose and the glucose detected by printing on paper and detection with ammoniacal silver nitrate.

The isolation of proteins from the jelly was achieved by electrophorising the protein out of the band into a buffer solution. The slab of jelly is placed on moist cellophane

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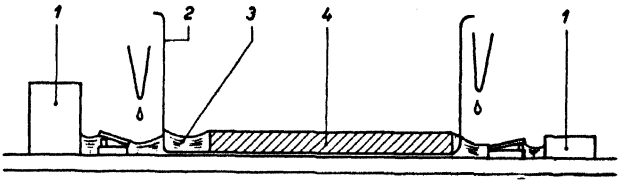

Fig. 70. Experimental arrangements suitable for migration of proteins out of agar jelly (Gordon *et al.* [6]).
1 electrodes
2 cellophane barrier
3 buffer
4 agar slab

for this process as shown in Fig. 70. The proteins cannot pass through the cellophane and can be pipetted out of the solution inside the cellophane. An observation of importance was made concerning the mobility of large molecules inside agar. The mobility in relation to that of a small molecule is in direct ratio to the agar concentration. Table 30 illustrates this relationship with ferritin, taking glycine for comparison. The consequences of this sieve-effect are discussed in Chapter 23. (See Table 30, page 185.)

Separation of proteins

Haemocyanin was considered of interest as it has one of the highest molecular weights known (6,600,000). In 0.09 *M* potassium phosphate buffer at pH 7 it travels 2.9 cm towards the cathode with 200 volts for 15 hours. The separation of *haemoglobin* and *ferritin* can be achieved with a 0.09 *M* potassium phosphate buffer at pH 7.6. *Egg white* proteins were separated, giving a pattern very similar to

TABLE 30

COMPARISON OF MOBILITIES OF A LARGE MOLECULE IN AGAR GELS OF VARYING CONCENTRATION IN 0.1 *M* ACETATE BUFFER AT pH 4.6 (IONIC STRENGTH 0.1) (Gordon *et al.*[6])

Concentration of agar %	Volt/amps	Duration of run hrs	Migration in cm		Ratio of migrations
			glycine	ferritin	
1	300/0.1	4	6.2	4.4	1.4
2	300/0.1	4.15	7.2	4.3	1.67
3	300/0.1	3	4.2	2.2	1.91
4	300/0.1	2	2.5	1.1	2.28

that obtained by free electrophoresis. The pattern was obtained by plotting the UV absorption curve. The two buffers employed were 0.09 *M* potassium phosphate at pH 6.8 and 0.13 *M* ammonium carbonate buffer at pH 8.0. *Plasma proteins* were successfully separated in 0.033 *M* potassium phosphate buffer at pH 7.9. The curve shown in Fig. 71 below gives the UV absorptions and *N* Kjeldahl figures along the gel. See also Bussard [15]. *Crude pepsin* was studied at pH 5.3 in 0.25 *M* sodium acetate buffer. 250 mg of crude pepsin were separated and the fractions recovered by electrophoresis inside cellophane[6].

Difficulties were always encountered in preparative work employing agar, in removing the last traces of agar from the fractions unless the protein was crystallised after separation. The mobile constituent of agar must also always be removed as it otherwise contaminates other substances on the anode side.

An interesting combination of agar electrophoresis and

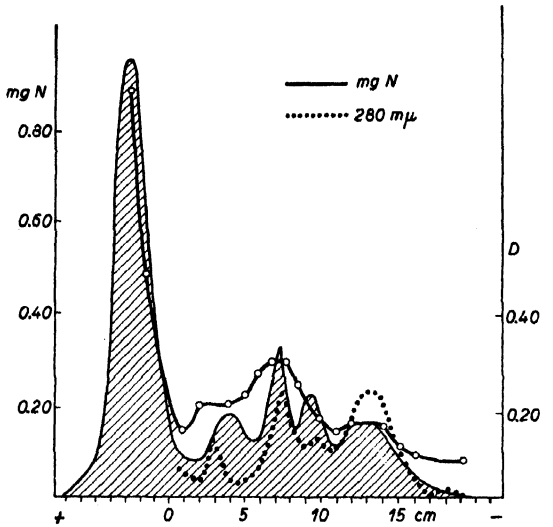

Fig. 71. Ultra-violet absorption and nitrogen content of jelly after electrophoresis of plasma compared with a similar analysis by the Tiselius method (solid curve) (Gordon *et al.* [6]).

immunological methods has been worked out by Grabar [16, 17]. Several papers already indicate numerous possible applications [18, 19].

Oligonucleotides

Gordon and Reichard [7] employed the technique originally developed for proteins, to the analysis of desoxyribonucleic acid (DNA) degradation products. The troughs used were 85 × 15 × 0.78 cm and for preparative work 60 × 60 × 0.78 cm. The technique of measuring the UV absorption of sections was again employed, but the agar

was dissolved in water before measuring in the spectrophotometer at 258 mμ. The agar was prepared as follows: 1 % agar 300 grams, 4 grams of NaOH and 21 grams of citric acid were brought to boil with 700 ml of water. This gives a gel of pH 3.7. 4 mg of fluorescein were added to this solution as this reveals the purine and pyrimidine bands

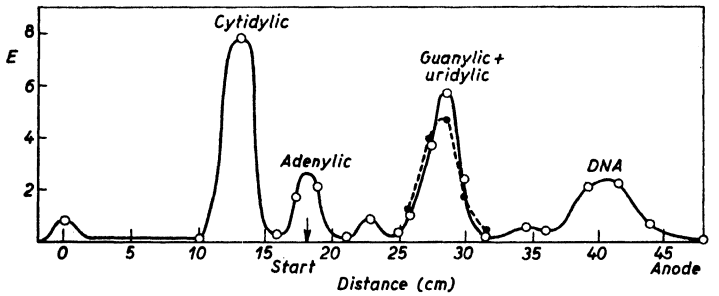

Fig. 72. Electrophoresis of desoxyribonucleic, adenylic, cytidylic, guanylic and uridylic acids at pH 3.7. Solid curve, light absorption at 262 mμ; broken curve, light absorption at 248 mμ (Gordon and Reichard [7]).

as non-fluorescent areas. The solution is allowed to cool to 60° and poured 0.78 cm thick. A gutter 5.0 × 0.9 cm and 20 cm from the cathode end is cut out. 3.5 ml of agar solution containing 50–100 mg of nucleotides are placed in the gutter. Fig. 72 shows the separation of an artificial mixture of 10 mg DNA, 10 mg adenylic acid, 10 mg cytidylic acid, 10 mg guanylic acid and 10 mg sodium uridylate run for 2 hours at 2.3 volt/cm, then for 15 hours at 3.7 volt/cm. Several enzymic hydrolysates of DNA were separated employing this technique.

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Porphyrin pigments

A method using a tube filled with agar instead of slabs was employed by Papastamatis and Kench [8]. The agar was purified to remove metal traces by washing with pH 8 Soerensen buffer. 0.5–1.0 % agar with *M*/15 potassium phosphate buffer at pH 8 was used in tubes 50–80 cm long with a potential of 4 volt/cm. The tube was surrounded by a water jacket to avoid the melting of the agar gel. In this work the sample was introduced by means of a side arm in the electrophoresis tube. The porphyrins could be separated into those with 2 COOH, 4 COOH and 8 COOH groups. The separation of the two coproporphyrins I and III was only successful in a tube 80 cm long.

Phenols and phenol condensation products

Another method using tubes filled with agar was used by Peniston, Agar and McCarthy [9]. The agar was purified by washing with distilled water and 1 % solutions prepared. The most commonly used buffers were pH 4.6 sodium acetate 0.06 or 0.12 M or a phosphate buffer at pH 7 (0.06 *M*). The tube was filled by pouring liquid agar to a certain level, cooling, then pouring in the sample, allowing that to cool and finally filling the tube to the top with buffer agar. The bands were detected after separation by scanning the ultra-violet absorption directly on the tube with a spectrophotometer in 2.5 mm or 10 mm intervals. The following separations are recorded: vanillin – vanillic acid and ferulic acid with 0.12 *M* acetic acid – 0.12 *M* sodi-

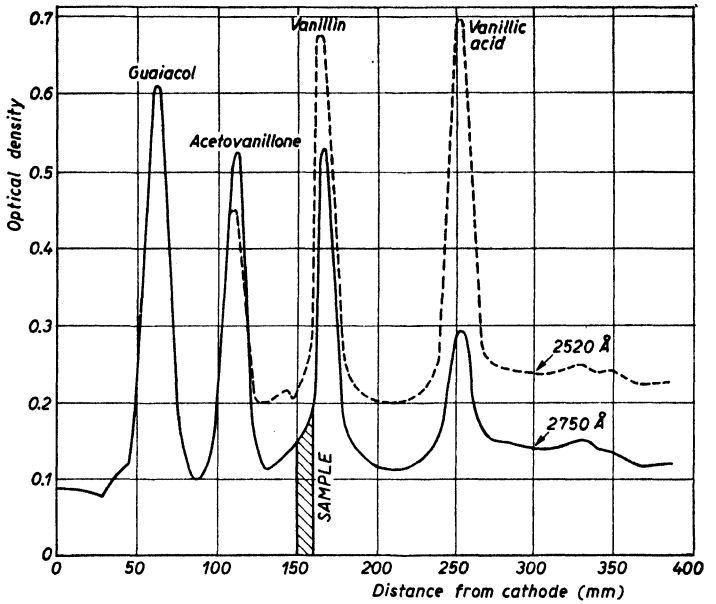

Fig. 73. Separation of phenolic substances (Peniston *et al.* [9]).

um acetate for 3.5 hours at 10° in a water bath with 390 volt (6.73 volt/cm) in a tube 76.1 cm long. The separation of guaiacol, acetovanillone, vanillin and vanillic acid with pH 7 phosphate buffer is shown in Fig. 73. Poly condensation products of formaldehyde with *p*-phenolsulphonic acid were also separated.

Antibiotics

A simplified slab-technique used directly on agar culture plates was worked out by Ludford and Lederer [10]. By

electrophorising inside the nutrient agar, it is possible to separate and compare antibiotics produced by various strains of bacteria without any isolation procedure. To keep sterile conditions during electrophoresis the following procedure was adopted: Two strains were tested together on one plate. Heart infusion agar in 35 ml amounts was poured into 15 cm petri plates and allowed to harden. Excess agar was then excised with a sterile knife to leave a strip 5 cm wide across the diameter of the plate. The strains under test (*Escherichia coli*) were then placed halfway along the strip in 2–3 mm inocula, a few millimetres from the edge, and the plate incubated overnight at 37° C. The organisms were then killed by exposure for 30 minutes at 37° to chloroform placed in the lid of the plate. Excess chloroform was removed by inversion of the two parts of the plate in the incubator for fifteen minutes. The plate, with the lid removed, was then inverted on two blocks of heart infusion agar standing in petri dishes containing 10–15 ml of Soerensen's phosphate buffer of pH 7.4. Carbon electrodes connected to a 45 volt Radio B battery were then placed in the petri dishes. To prevent overheating of the agar, a tin containing ice was placed on the plate containing the strip. With potentials higher than 45 volt, this cooling device was inadequate, and cracking and dehydration of the agar occurred. Electrophoresis was usually carried out for five hours, as dehydration increased if this period was extended and in a longer time the faster moving components possibly would have run over the

strip. When electrophoresis was concluded, the plate, with the lid removed, was inverted in the incubator for a few minutes to remove any condensed moisture. The agar strip was again exposed to chloroform vapour for approximately fifteen minutes in an attempt to prevent contamination. Excess chloroform was removed as described earlier.

Finally, 0.8 ml of a 1 in 5,000 dilution of a 24 hour

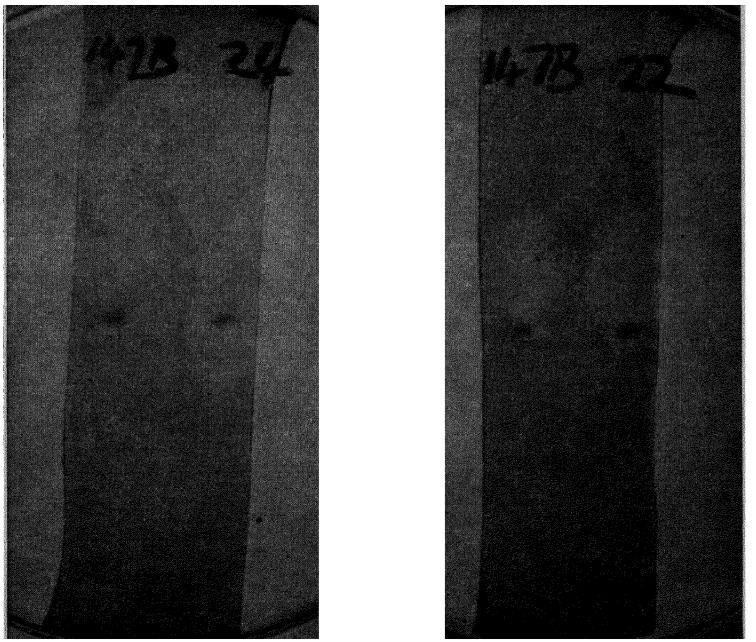

Fig. 74. Electrophoresis of antibiotics in agar gel. Both figures show the separation of the antibiotics of different strains of *E.coli* electrophoresis being performed inside the nutrient agar and the "spots" revealed with *Shigella sonnei* (Ludford and Lederer [10]).

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broth culture of the indicator *Shigella sonnei* strain was run on the strip and spread with a Pt wire. After incubation overnight the spots of antibiotic are revealed as clear zones, as shown in Fig. 74. For the separation of the B vitamins with microbiological detection see Marten [20].

Inorganic compounds

The work of Kendall *et al.* [11–13] with tubes of gels 3 metres long and 4–7 cm in diameter is one of the first analytical applications of ionic migration. Pairs of rare earths, halogens, Zr–Hf and other metals were examined; the reader is referred to the original literature for details. Lederer and Cook [14] separated some pairs of metals inside slabs of 2 % agar containing as electrolyte *N*/2 NaCl. Agar can not however be used with acid electrolytes, as it does not gel in mineral acid solutions. As a preparative method it may find uses, for example in noble metal separations.

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22. Measurement of Diffusion Rates Inside Porous Media

Although measurement of diffusion rates is neither an analytical technique nor connected with migration in an electric field, a brief survey of the diffusion methods inside porous media will be given, as many have been worked out simultaneously with electrophoretic methods. Diffusion constants also help in some cases to identify macromolecules after electrophoretic separation.

Brooks and Badger [1] used stacks of filter paper sheets as the porous medium in the differentiation of nitrocellulose fractions of varying degrees of polymerisation. They placed one sheet of filter paper containing the solute, and saturated with the solvent, on top of a pad of 100 solvent-saturated filter papers, and, by permitting diffusion to go on for a known time, obtained a three-dimensional diffusion rate through the pad; the measurement of diffusion was carried out by separating the filter paper sheets and detecting the solute by spot tests.

It is also possible to obtain relative diffusion rates by impregnating paper sheets with the solvent and placing on them spots of known volume and concentration as for an electrophoretic separation, then allowing the spots to diffuse in a saturated atmosphere and subsequently evaluating by dyeing. This method was worked out by Lederer[2] to obtain the order of spot size increase during an

electrophoretic run. The accuracy of such measurements is the same as for spot area determination in paper chromatography (i.e. $\pm$ 5 %) and thus diffusion can be measured easily whenever the substances do not adsorb strongly on the paper.

In their work on oligonucleotides Gordon and Reichard [3] measure the diffusion constants of the isolated oligonucleotides in the following manner: The sections of agar from the electrophoretic separation (see page 186) are made up to 30 ml with 750 g/l in 0.1 *M* sodium citrate and allowed to solidify in flat-bottomed bottles. The agar block so formed is held with a volume of supernatant isotonic citrate solution at 27° C for 1, 2, 3, 4 and 5 hours, and the amount of oligonucleotide diffused out of the agar block determined spectrophotometrically. Thus comparative diffusion constants were obtained for the various separated fractions and di- and mononucleotides could be differentiated by their diffusion rates.

Peniston, Agar and McCarthy [4] measure the diffusion rates of polyphenols inside agar. They scan directly the tube containing the agar by spectrophotometry. The diffusion constants were calculated from the shape of the curves obtained.

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23. Electrokinetic Ultrafiltration

This method developed by Mould and Synge [1] utilises filtration inside a strip of a collodion membrane in the same way as in chromatography, but with the pore size governing the rate of movement of the substances and not the adsorption or partition. The "development" stage is performed by passing liquid through the length of the membrane by electro-osmosis.

Successful separations of polysaccharides consisting of glucose residues in a α–1 :4-linkage were reported. The material examined was a series of enzyme-synthesised polysaccharide preparations made by allowing potato phosphorylase to act on known amounts of pure maltohexaose in presence of glucose-1-phosphate. From the amount of inorganic phosphate liberated in the reaction, it was possible to calculate the average DP (degree of polymerisation) of the synthesised polysaccharide.

The membranes used were strips of collodion ultrafiltration membranes of different porosities, prepared according to the general directions of Elford [2] and kept in 0.2 *N* acetic acid. The strip, measuring 1.3 cm × 8.0 cm, was mounted vertically in a wide test tube, through the bottom of which a platinum-wire cathode was sealed. The 0.2 *N* acetic acid surrounded the cathode, and the bottom end of the strip dipped into this catholyte. The greater

part of the strip was immersed in liquid paraffin, B.P., overlying the catholyte in the test tube, but the cut surface of its top end was in contact with 0.1 to 0.2 ml of the anolyte, which was held in a chamber sealed with rubber gaskets between two microscope slides whose ends gripped the top end of the strip. A platinum wire anode dipped into the anolyte. A solution of the polysaccharide mixture for analysis, approximately 1 per cent. w/v in dilute acetic acid, served at first as anolyte. On applying 250 volts to the electrodes, this solution entered the strip by electro-osmosis. While maintaining this potential, the anode chamber was then perfused continuously with 0.2 *N* acetic acid; this stage corresponds with elution development of the chromatogram with fresh solvent. After a suitable time, a strip was removed from the apparatus and sprayed with iodine-potassium iodide solution. This immediately revealed the positions of the polysaccharide zones which were often completely resolved from one another (see Table 31).

For the measurement of R_F values fructose was added to the solution which, as has been demonstrated, is not retained and thus is at the "liquid front". The fructose was detected by cutting one half of the strip in portions and detecting the presence of fructose in aqueous extracts of the portions by the "rapid furfural" test. For further work see also Mould and Synge [3].

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TABLE 31

R_F VALUES OF POLYSACCHARIDES WITH MEMBRANES OF DIFFERING POROSITIES

Membrane	Pore radius	R_F for polysaccharides of average DP							
		34.6	47.5	77	103	145	160	205	250
A	530	1	1	–	0.84	0.78	–	0.47–0.74	0.30–0.59
B	29	0.62–0.82	–	0.35–0.55	0.21–0.45	–	0.07–0.16	0	0
C	8.6	0.39–0.56	0.31–0.48	0.15–0.34	0.08–0.18	0	0	–	–
D	7.9	0.27–0.49	–	0.10–0.20	0	0	0	0	0

Note. Where two figures are given, these signify R_F values of extreme limits of zone width observed in whole series of experiments. Single figures refer to R_F of zone centres.

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(For pure elements see symbols)